

**IMMUNOMODULATORY EFFECTS OF PROBIOTIC AND ANTICOCCIDIAL
TREATMENTS IN BROILER CHICKENS**

A Dissertation

by

KENDRE DUARON STRINGFELLOW

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2012

Major Subject: Poultry Science

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ABSTRACT

Immunomodulatory Effects of Probiotic and Anticoccidial Treatments in Broiler
Chickens.

(August 2012)

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Four experiments evaluated the impact of probiotic administration on the immune response of broilers vaccinated with a live coccidiosis vaccine. Experiment one showed that probiotic administration increased heterophil and monocyte oxidative burst, and lymphocyte proliferation at multiple time points. In experiment two, probiotic + vaccine increased heterophil and monocyte oxidative burst on d 15 when compared with the negative controls. Overall, vaccine administration alone showed the highest response when compared to all other treatments. In the second trial, all birds were exposed to *Eimeria* oocysts in the litter and oral gavaged. The results showed that probiotic + vaccine resulted in greater heterophil and monocyte oxidative burst levels on d 14 and 28 when compared to the negative controls. Increases in lymphocyte proliferation were also seen in the probiotic + vaccine and probiotic alone broilers on d 14 among other treatments.

In experiment three, heterophil oxidative burst was increased ($p \leq 0.05$) in the vaccine alone group, vaccine with probiotic group, and the ionophore with probiotic group, when compared to the negative control. Monocyte oxidative burst was increased ($p \leq 0.05$) in the vaccine with probiotic group on d 36 and 43, compared to the negative control. Lymphocyte proliferation was greater ($p \leq 0.05$) on d 22 and 36 in the ionophore with probiotic group, when compared to the negative control.

Experiment four showed that liver AVBD 2 gene expression elevated ($p \leq 0.05$) in the probiotic + vaccine group relative to the probiotic alone group. Ileum AVBD 2 gene expression was not affected among any of the treatments was evaluated. Liver AVBD 9 was demonstrated to have higher ($p \leq 0.05$) gene expression in the vaccine group when compared to controls. When AVBD 9 gene expression was evaluated in the ileum, a decrease ($p \leq 0.05$) was observed in all treatments compared to the control group. These data suggest that simultaneous administration of probiotics during coccidiosis vaccination or ionophore treatment has the ability to modulate the immune response at varying time points.

DEDICATION

First and foremost I would like to thank the good Lord for giving me the opportunity to study at Texas A&M and for the continued support, love, and strength he has given me.

This dissertation is dedicated to my caring and devoted mother, Mrs. Debra Stringfellow, for all your love, support and encouragement. Despite all the obstacles in your way, you managed to transform me from a young immature child into a responsible adult.

This dissertation is also dedicated to my late father, Mr. Larry Stringfellow. Although you are not able to be physically present to enjoy this opportunity, I know that you are watching over me.

To my brothers, Kendric and Kwame Stringfellow and my sister, Kiaa Stringfellow, I appreciate your continued moral support and sound advice. If it were not for your continuous love and motivation, I would not be where I am today.

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CHAPTER I

INTRODUCTION

Avian coccidiosis, commonly caused by the enteric parasite *Eimeria*, is a widespread disease that negatively affects the world's poultry industry (Williams, 2003). Nine species of *Eimeria* have been described in chickens, but only seven of those species have been found to be pathogenic: *Eimeria acervulina*, *E. brunetti*, *E. maxima*, *E. tenella*, *E. praecox*, *E. mivati* and *E. necatrix* (Mcdougald, 2003). It was estimated that the total worldwide cost of avian coccidiosis was \$3 billion, with U. S. losses totaling \$800 million (Williams, 1999). Factors attributing to this estimate include the cost of prophylactic in-feed medication, alternative treatments, increased mortality and morbidity and poor feed conversion of infected birds (Williams, 1999).

To reduce the risk of contracting an infection, the U. S. poultry industry has largely relied on prophylactic in-feed medication (Williams, 2005). These drugs are classified as a chemical- type, which adversely affects parasite metabolism, or as an ionophore-type, which alter parasite ion transport and osmotic balance (Allen and Fetterer, 2002). Due to the development of drug resistance by *Eimeria* field strains and the consumer concern for drug residues in poultry products, there has been a shift from using in-feed drugs, to the use of vaccines (Chapman et al., 2002; Williams, 2002).

The use of live vaccines for coccidiosis control in broiler breeder or layer chickens is well established (Chapman et al., 2002; Williams, 2002). However, in broilers, the perceived negative effects on early chick growth and concerns about the timely onset of protective immunity has caused poultry producers to be reluctant in using

This dissertation follows the style of Poultry Science.

vaccines due to their relatively short production cycle of 6 - 8 weeks (Danforth, 1998; Allen and Fetterer, 2002; Williams, 2002). In a comprehensive review of the literature analyzing comparative trials between anticoccidial vaccines and drugs, Williams (2002) reported that there were few consistent differences between the performance (daily weight gains, adjusted feed conversion ratios and mortality) of either vaccinated broilers or broilers receiving anticoccidial drugs. Other reports have indicated that vaccinated broilers have a significantly lower mortality rate, when compared to broilers treated with anticoccidials (Williams, 1999; Williams, 2002). The development of protective immunity largely relies on subsequent reinfections by the ingestion of *Eimeria* oocysts in the litter (Vermeulen et al., 2001); as demonstrated by Chapman et al. (2002), vaccinated birds reared in cages remained susceptible to an infection when compared to floor reared birds (Chapman et al., 2002).

There are two primary forms of vaccines used in commercial poultry, non-attenuated live vaccines which include *Eimeria* field strains that have not been modified to affect their virulence (Coccivac[®], Immunocox[®]) or attenuated live vaccines that contain *Eimeria* that have been either selected for precocity or passed through embryonated hens' eggs (Paracox[®], Livacox[®]) (Danforth, 1998; Williams, 2002). One drawback of using live vaccines is that vaccination occurs within the first d of the neonate's life when the adaptive immune system is functionally immature (Vermeulen et al., 2001). Therefore, the risk of contracting coccidiosis or secondary infections caused by resident opportunistic bacteria is increased during this time, but decreases as immunity is established (Vermeulen et al., 2001).

Potentiating the immune system with probiotics can reduce *Eimeria* colonization (Dalloul et al., 2003) and may increase protection within the early days following vaccination and throughout the production cycle, minimizing the onset of disease. Probiotics have been shown to enhance gut health, and potentiate innate and adaptive immunity (Koenen et al., 2004; La Ragione et al., 2004; Farnell et al., 2006). Yurong et al. (2005) found an increase of immunoglobulin (Ig) A in the intestinal fluid, IgG and IgM secreting B-cells in the Peyer's patches and increased T-cell numbers in the cecal tonsils of probiotic treated chickens, indicating that probiotics may enhance intestinal mucosal immunity. Additionally, Dalloul et al. (2005) demonstrated enhanced mucosal immunity against *Eimeria acervulina* in broilers fed *Lactobacillus*-based probiotics. These researchers found an increase in gamma interferon and interleukin (IL) 2, which are both involved in protective immunity against an *Eimeria* infection (Yun et al., 2000; Lillehoj et al., 2004). Interleukin 2 is a growth factor for an array of cell-types, including T -and B-cells, while gamma interferon is traditionally considered as a macrophage-activating factor (Lillehoj et al., 1992). A reduction in fecal oocyst shedding was also observed, which positively correlates to increased resistance of the host to *Eimeria* (Dalloul et al., 2003). One of the objectives of the present research was to determine if probiotic bacteria can similarly influence the avian mucosal immune system in vaccinated or ionophore-treated broilers by analyzing heterophil, monocyte and T-cell activity.

Human defensins have been reported to mediate inflammation, activate leukocyte chemotaxis, promote wound healing and macrophage response (Hancock and Diamond,

2000; Scott and Hancock, 2000; Ganz, 2003). Avian Beta-defensins (**AVBD**), originally termed gallinacins, were first isolated from chicken heterophils (Evans et al., 1994; Harwig et al., 1994). The exact antimicrobial activity of AVBD in poultry is unknown; however defensins in humans, plants, flies and mammals have been speculated to cause membrane disruption of invading microbes (Kagan et al., 1990; Satchell et al., 2003). Avian Beta-defensins have a broad spectrum of antimicrobial activity against bacteria, protozoa, fungi and enveloped virus (Donovan and Topley, 2003). The avian heterophil, a leukocyte similar to the mammalian neutrophil, lack superoxide ion and myeloperoxidase and therefore mainly rely on non-oxidative mechanisms for pathogen defense, which include AVBD (Maxwell and Robertson, 1998). The expression of AVBD is expected to increase in the presence of an antigen such as lipopolysaccharides, a Gram - negative bacterial cell wall component (Abdel-Mageed et al., 2008). There are currently 14 AVBD that have been recognized in a variety of immune tissues (Xiao et al., 2004). To further evaluate the immunomodulating effects of probiotic administration in vaccinated or ionophore-treated broilers, this research analyzed AVBD 2 and 9 gene expressions in the liver and ileum.

CHAPTER II

LITERATURE REVIEW

Avian Immune System

Innate Immunity

The immune system of the chicken starts developing *in-ovo* and continues to develop throughout its life (Janeway et al., 2001). The avian immune system is subdivided into two systems that ultimately work in concert with each other to facilitate pathogen clearance following a microbial infection. These two systems are termed innate and adaptive immunity, with each having different components and responses to pathogen invasion of the host (McDonald, 1999). The innate immune system is comprised of constitutive barriers including the skin, fatty acids found on the skin, mucus and mucus secretion of the mucosa, pH levels in the gastrointestinal environment and ciliary movement in the trachea (Juul-Maden et al., 2008). Lysozyme and antimicrobial peptide secretions of epithelial cells, inflammation, the activation of the complement system and resident microflora also play a key role in innate defense mechanisms (Matsushita and Fujita, 1992). In addition to the physical barriers, leukocytes are the primary effector molecules in innate defense mechanisms and include natural killer cells, macrophages, heterophils, mast cells and dendritic cells (Beutler, 2004). These cell types are the first responders to pathogen infection and are able to mount an immune response early in the chick's life because their antigen recognition molecules are encoded in the germline DNA and are therefore present at hatch (Beutler, 2004). These cells utilize pathogen recognition receptors (**PRR**) to identify pathogen

associated molecular patterns (**PAMPS**) on invading microorganisms. Some PRR that have been identified are membrane toll-like receptors that recognize bacterial peptidoglycan (TL1/6/10), the GPI anchor of parasites such as *Eimeria* (TL2), double stranded RNA (TL3), lipopolysaccharides (TL4), flagellin (TL5), viral single stranded RNA (TL7/8), and a bacterial CpG motif (TL9) (LeMaitre et al., 1996).

Following tissue injury or injury due to an invading microbe, inflammation occurs. Inflammation consists of a large number of systemic and metabolic changes, referred to as the acute phase response (Janeway, 2001). The acute phase response causes fever, anorexia, increased synthesis of a number of hormones and proteins, inhibition of lipid metabolism, and an increase in plasma proteins. Acute phase proteins are proteins whose plasma concentrations increase 25% or more within 4-24 hr following inflammation initiation (Juul-Maden et al., 2008). Acute phase proteins (**APP**) include C-reactive protein (Pepys and Hirschfield, 2003) and mannan binding lectin (Turner, 1996) which bind to foreign antigen and damaged host cells facilitating their removal via phagocytosis. Another group of APP include fibrinogen and fibronectin which are responsible for tissue repair, homeostasis, cellular growth and differentiation (Mathews and van Holde, 1990) and serpins (serine protease inhibitors) which protect the host from serine proteases that cause injury due to its production of harmful enzymes that are able to cause tissue damage (Beutler, 2004).

Inflammation also promotes the recruitment of leukocytes and extravasation of several plasma proteins to the site of infection and activation of leukocytes and plasma proteins to eliminate the invading agent. Inflammation and APP also promote the

activation of the complement system. The complement system consists of over 25 plasma proteins that are constitutively expressed in an inactive form (Matsushita and Fujita, 1992). Once the proteins recognize cellular components of invading microorganisms, they become sequentially activated. The effector mechanisms of this system include enhancing phagocytosis by opsonization, induction of an inflammatory response, enhancing B- and T-cell responses and cytolysis via a membrane attack complex (Thiel et al., 1997). There are three pathways: classical (activated by IG-antigen complex following a humoral response), lectin pathway (activated by lectin recognition of PAMPS) and an alternative pathway (C3b bind to microbial cell walls (polysaccharides, lipopolysaccharides (**LPS**)) (Abbas and Lichtman, 2003).

Natural killer cells are thymus-independent and therefore belong to the innate immune system however they share some homology with the cytotoxic T-cell due to the presence of a surface CD8 molecule but differ in that a CD3 molecule is not co-expressed (costimulation of CD3 is required for cytotoxic T-cell; Sharma, 1997). Natural killer cells achieve destruction of invading microbes by facilitating cell-mediated cytotoxicity, which is the release of cytoplasmic granules (perforin and granzymes) that impair the cell-membrane of the infected cell. Natural killer cells are the main effectors in tumor and virally infected cells and recognize the infected cell following antigen presentation in association with its major histocompatibility complex I (**MHCI**; Janeway, 2001). Cytokines, released by the infective cells, include IL 2 (proliferative cytokine) and IL 12 and act to further stimulate the killing activity of natural killer cells (Janeway, 2001). Natural killer cells also have an Fc-receptor that is

used to bind to antibody-antigen complexes and once activated will participate in antibody dependent cellular cytotoxicity activity (Chi and Thorbecke, 1981).

Macrophages are cells that serve very important roles in both innate and adaptive immunity including modulation of the innate and adaptive immune system, tissue homeostasis and pathogen recognition (Gregory and Devitt, 2004). Macrophages are found in various tissues throughout the host and are derived from blood circulating monocytes (Gregory and Devitt, 2004). Once monocytes are attracted to their resident tissue via specific chemotactic signals and migrate deep into the tissue via high endothelial venule adhesion molecules, they are re-defined as macrophages (Horino et al., 1998). Due to their close association with tissues, these cells are one of the first responders of the immune response. One of the primary defense mechanisms of macrophages is phagocytosis. During phagocytosis the fragments from invading microbe are internalized into phagosomes and subsequently fused with lysosomes which form a phagolysosome (Szondy et al., 2003). Destruction ensues from lysozymic enzymes (acid phosphatase and β glucuronidase) and oxidative burst which is the release of reactive oxygen and nitrogen intermediates (Szondy et al., 2003).

Following phagocytosis, the antigenic fragments of the ingested and degraded microbe are then bound to MHCII and presented on the surface for recognition by a T-cell receptor on resident T-cells. Following T-cell recognition, the T-cell may either produce Th1 or Th2 cells, with each having its own respective cytokine profile (Abbass and Lichtman, 2003). Thymus-derived Th1 cells may secrete gamma interferon, which upregulates phagocytic activity while Th2 may secrete IL 4, 5 and 10 which will induce

a humoral immune response (Fadok, et al., 2001). Macrophages will also produce nitric oxide which has a static effect on bacterial growth and IL 1 and 6 and tumor necrosis factor which are potent inflammatory cytokines (Fadok et al., 2001).

Similar to macrophages, heterophils are also capable of phagocytosis and are the first responders to an inflammatory stimulus (30 minutes; Maxwell and Robertson, 1998). Heterophils outnumber lymphocytes in chicks between hatch and one week of age with their numbers increasing during stressful conditions (Maxwell and Robertson, 1998). Heterophils are the counterpart of mammalian neutrophils and have also been stated to be the first line of cellular defense against invading pathogens (Janeway and Medzhitiv, 2002). A distinguishing factor is that heterophils lack myeloperoxidase which reduces the efficacy of oxidative burst granules in comparison with neutrophils that are capable of producing the highly bactericidal hypochlorous acid, generated from hydrogen peroxide, an oxygen free radical (Powell, 1987). Their cytoplasmic granules contain several lysosomal and non-lysosomal enzymes including acid phosphatase, arylsulphatase, β -glucuronidase, phosphorylase, uridine diphosphate glucose-glucogen glycosyltransferase, neutral and acid α -glucosidases, acid trimetaphosphatase and lysozyme (Maxwell and Robertson, 1998).

Dendritic cells are considered the most potent antigen presenting cells when compared to macrophages and B-cells. These cells play important roles in antigen capture and the induction of T-cell responses (Schrama et al., 2002). Dendritic cells are found under epithelium and in most organs (Schrama et al., 2002). Dendritic cells capture foreign antigen and transport these antigens to peripheral lymphoid organs. In

peripheral organs dendritic cells migrate to the interfollicular T-cell dependent zones and present antigen to naïve T-cells in association with the MHCII complex (Schrama et al., 2002). Following antigen-MHCII complex interaction with the T-cell receptor and accessory co-stimulatory molecules, T-cell clonal expansion and differentiation occurs (Janeway, 2001).

Mast cells are potent promoters of inflammation and mediate immediate hypersensitivity reactions and are found near the portal entryway of many microorganisms including the skin, mucosal surfaces and around blood vessels (Abraham and Malaviya, 1997). Benefits of mast cells include phagocytosis and killing of microbes, recruitment of immune cells, presentation of microbial antigens to immune cells and regulation of other adaptive immune responses (Abraham and Arock, 1998). Mast cells are primarily activated by two mechanisms including opsonin-dependent and -independent recognition (Abraham and Arock, 1998). Opsonin- dependent activity takes place following opsonization of the microorganism prior to mast cell recognition versus mast cell interaction with the complimentary ligand on a bacterial cell surface as seen in opsonin-independent occurrences (Beutler, 2004). Upon binding the receptor, the mast cell releases histamines which increase vascular permeability, prostaglandins which cause vasodilation and act as a heterophil chemoattractant, and leukotrienes which facilitate mucus secretions and tumor necrosis factor α and IL 4 (Abass and Lichtman, 2003). Upon IgE binding, mast cells will also release cytoplasmic granules and peroxidases that are toxic and capable of degrading microbial cell wall components (Abass and Lichtman, 2003).

Cells of the adaptive system are not fully functional at hatch and do not completely mature until about two weeks post-hatch (Sharma, 1997). Therefore the neonate primarily relies on the innate system for early protection against pathogens. The adaptive system comprises both B and T lymphocytes and these cells rely on maturational processes that take place in the primary lymphoid organs, bursa of Fabricius and the thymus, respectively (Cooper et al., 1965). The bursa is located in the proctodeum region of the avian cloaca and is responsible for B-cell maturation (Cooper et al., 1965). This structure is unique to the avian species and has contributed to the mammalian nomenclature of B-cells derived from bone marrow (Cooper et al., 1965). The thymus is located in the neck parallel to the jugular vein and is composed of about 7-8 lobes on each side (Sharma, 1997). Features that distinguish adaptive from innate immunity include differences in specificity, diversity and memory (Abass and Litchman, 2003).

As stated previously, cells belonging to the innate branch of the immune system use PRR to recognize various components of different microorganisms. Their pattern recognition capability does not adapt to or change to subsequent changes in microbial molecular structures or novel pathogens. In contrast, the cells of the adaptive system undergo changes to generate genetic diversities of the T-cell receptor and B-cell receptor through manipulation of their genes during their development (Kincade et al., 1971). Each membrane B- or T-cell receptor will recognize only one antigenic epitope (Janeway, 2001). In contrast to mammals, which contain two light chain locus and use gene rearrangement to generate light chain diversity, avian species have a single light

chain loci and use somatic gene conversion (Fugmann et al., 2000). This differs from the avian species in that there are pseudogenes present during gene conversion and these are thought to contribute to the light chain specificity (Arnon et al., 2008). It's been speculated that this same conversion of genes apply to heavy chain specificity (Arnon et al., 2008). Mammals have large families of heavy chain variable, diversity and joining gene segments, whereas in the chicken single variable and joining regions recombine with a family of diversity gene segments (Rudolph et al., 2006).

Initial exposure of the immune system to a foreign antigen will enhance its ability to respond to that antigen during a subsequent encounter. The response to the second and subsequent exposures will be more rapid, increased, and cell types will be more specific from the first exposure (Abass and Lichman, 2003). The stimulation of naïve lymphocytes will produce memory cells and well as effector cells. Memory cells have special characteristics that make them more efficient at eliminating the pathogen when compared to naïve lymphocytes. Such is the case for naïve mature B-cells whose secreted immunoglobulins bind with less affinity when compared to the secreted immunoglobulins from memory B-cells (Abbass and Lichman, 2003). Naïve mature T-cells will migrate more slowly to the site of infection when compared to memory T-cells (Abbass and Lichman, 2003). This observation has led to the development and success of vaccine administration in both mammal and avian species (Shirley et al., 2005).

Adaptive Immunity

Humoral immunity is mediated by secreted antibodies, which are produced by the activated B-cell (Kincade et al., 1971). Antibodies bind to the antigens of extracellular microbes and function to neutralize and eliminate these microbes. Antibody response to protein antigens requires the cooperation with CD4 Th cells that recognize the antigen and facilitate further activation of the B-cell via MHCII (Kaufman et al., 1999). Antibody responses to non-protein antigens such as lipid and polysaccharides do not require a subsequent encounter with T-cells or MHCII bearing cells. After activation, activated B-cells differentiate into antibody secreting B-cells as well as memory cells that are circulating for subsequent encounters of the same antigen. Heavy chain isotype switching and affinity maturation occur following successful activation of the B-cell. Once activated, antibodies participate in neutralization of microbes and toxins via opsonization which enhances phagocytic activity, antibody dependent cell cytotoxicity and complement activation (Abbass and Liethman, 2003).

Cell-mediated immunity is mediated by the activation of T-cells. The adaptive immune response to microbes that infect and replicate within the cytoplasm of various cell types is mediated by CD8 cytotoxic T-cell which kill the infected cells and eliminate the reservoirs of infection. The adaptive immune response to parasites is mediated by Th2 cells, which stimulate the production of specific antibodies and activate.

Eimeria

Avian coccidiosis is a prevalent disease affecting the world poultry industry. The economic losses from coccidiosis are greater in chickens when compared to other domesticated fowl (Reid et al., 1976). Coccidiosis affects the intestines of poultry and is caused by parasites of the genus *Eimeria* (Levine, 1982). *Eimeria* are protozoa within the taxonomic family Eimeriidae and the phylum Apicomplexa (Levine, 1982). Seven species of *Eimeria* have been identified to cause disease in commercially reared chickens, including *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. praecox*, *E. necatrix* and *E. tenella* (McDougald et al., 1997). *Eimeria acervulina* sporozoites migrate from the lumen and develop in epithelial cells of the duodenum and upper jejunum (Reid et al., 1976). The oocysts are egg shaped and have visible thinning of the shell at the smaller end, with an elevation termed the micropyle (Reid et al., 1976). *Eimeria brunetti* produced lesions in the ileum, large intestine, and proximal areas of the ceca (Levine, 1973). Necrosis appears around 4-7 d following sporulation, producing an eroded surface covering the mucosa regions (Levine, 1973). *Eimeria maxima* epithelial cell invasion occurs in the middle of the small intestine above and below the yolk sac diverticulum (Meckel's diverticulum), as well as other areas of the intestine (Shirley et al., 1983). Hemorrhagic enteritis, thickening of the intestinal wall and some ballooning may be visualized following infection, with viscous and discolored intestinal contents (Shirley et al., 1983). *Eimeria mitis* does not produce gross lesions; slightly tapering and small oocysts are indicative of infection by this species (Tyzzer, 1929). Similarly, *Eimeria praecox* produces no gross lesions following migration in to the epithelium of

the upper third of the digestive tract (Joyner, 1982). Around d 4-5 of infection, small hemorrhaging of the mucosal surfaces can be seen with watery intestinal contents appearing on d 3 (Joyner, 1982). *Eimeria necatrix* migrates to the midgut near Meckel's diverticulum and produces lesions that may extend the length of the digestive tract (Joyner, 1982). Infection by *Eimeria tenella* has been termed cecal or "bloody" coccidiosis due to the invasion of the ceca and nearby areas of the digestive tract (Williams, 1999). Each species can cause disease but the clinical signs, symptoms and pathogenicity may vary (Williams, 1999). For example, infection caused by *E. mitis* or *E. praecox* will cause only reduced growth rate and feed conversions while infection by *E. necatrix* or *E. tenella* may result in a higher rate of mortality (Williams, 1998). *Eimeria acervulina*, *E. maxima* and *E. tenella* are more prevalent species that infect commercial poultry meat production (McDougald et al., 1997). However, all species may affect hen layer production (Williams, 1999). A 70% reduction in layer egg production was observed following a challenge with *E. acervulina*, *E. brunetti*, *E. maxima* and *E. tenella* within 3 weeks post inoculation (Reid, 1976).

It's been stated that zone of intestine parasitized, nature of macroscopic lesions, oocyst size, shape and color, schizont and area in which the parasite develop, location of the parasite within the epithelial cells, minimum prepatent period, minimum sporulation time, and cross-immunization trials are useful characteristics that would be helpful in identifying *Eimeria* species (Reid et al., 1976). Several molecular approaches have also been used to distinguish between species. Isoenzyme patterns by starch block electrophoresis was the first technique developed (Shirley et. al., 2005), followed by

using rRNA and rDNA probes, randomly amplified polymorphic DNA assays, recombinant DNA techniques and polymerase chain amplification (Allen and Fetterer, 2002).

Species of *Eimeria* are transmitted via the fecal-oral route (Williams, 1999). The severity of infection is ultimately determined with the number of infective oocysts ingested by the chicken (Johnson et al., 1986). Oocysts in the environment may be transported by definitive hosts, asymptomatic avian hosts, rodents, flying insects, other invertebrate pests, contaminated feed, old litter, humans and other equipment used in the rearing facility (Johnson et al., 1986).

The ability of the *Eimeria* species to thrive in an environment depends on the characteristics of the species, ubiquity of the species, climate and environmental conditions, sanitary conditions and the susceptibility of the breed of the birds (Williams 1999). Oxygen, moisture and warm litter are the ideal environment for excreted oocysts to sporulate (Williams, 2002). *Eimeria tenella* sporulation is optimal at 28°C, with a decline in sporulation rates beginning at 20°C and stops at 8°C (Reid et al., 1976). A lethal temperature of 55°C has been demonstrated to kill oocysts as well as freezing temperatures; however oocysts survival in the field is extended by the presence of high humidity and above freezing temperatures (Reid et al., 1976). Litter containing 31-62% moisture at 25 to 28 °C increased the sporulation rates of *E. acervulina* but this condition became lethal to the oocysts after 5 d (Williams, 1998). All seven species are ubiquitous and can occur on a farm in different combinations (Williams 1998). *Eimeria acervulina*,

E. maxima, *E. tenella*, *E. brunette*, *E. mitis* and *E. praecox* were present in litter samples within six weeks after a new flock placement (Williams, 2005).

The life cycle of *Eimeria* consists of an exogenous phase, which includes sporulation and an endogenous phase, which includes schizogony and gametogony (McDonald et al., 1999). Sporulation or differentiation occurs in the environment 24 hours following oocyst excretion in the feces causing them to express a number of antigens that are recognized by the chicken's immune system (Tomley, 1994). Each sporulated oocyst contains four sporocysts, with each sporocyte having two sporozoites (Allen and Fetterer, 2002). However, only a small percentage of oocysts will survive and be able to become infective (Reid et al., 1976). Following ingestion of the oocysts by the bird, trypsin, bile and carbon dioxide help the oocysts excyst within the intestinal lumen and undergo schizogony, which include three to four rounds of asexual reproduction (Allen and Fetterer, 2002). It takes 5-6 days to complete one sexual and two asexual cycles, with hemorrhaging occurring in the chicken around day 4, peaking around day 5 and cessation occurring around day 7 of the life cycle (Reid et al., 1976). Once the oocysts are ingested, the sporozoites emerge from the sporocytes in the lumen of the upper intestine and migrate to their respective site of development where they invade villus enterocytes. Villi atrophy occurs after infection, resulting in nutrient malabsorption (Fernando and McCraw, 1973). It's believed that the migration of the sporozoites to the crypts is also achieved via host intraepithelial lymphocytes (Fernando and McCraw, 1973) and macrophages due to their pinocytic ability (Trout and Lillehoj, 1993).

There are three forms of the disease with each having their own effects.

Coccidiasis is a mild infection causing no adverse effects (Levine, 1982), subclinical coccidiosis is described as causing reduced growth and increased feed conversion ratios (Williams, 2002), and clinical coccidiosis which causes frank hemorrhaging of the intestine (Levine, 1982). Signs of *Eimeria* invasion and disease include mortality, morbidity, diarrhea, bloody feces, reductions in weight gain, and increased feed conversion (Levine, 1982). Joyner (1982) reported that gut passage time of the digesta is decreased by *E. acervulina* infection. *Eimeria tenella* and *E. praecox* have also been demonstrated to reduce digesta viscosity in chickens, which directly affect performance parameters (Morgan and Catchpole, 1996).

Immunity to *Eimeria*

Immunity to *Eimeria* species depends on factors such as the host genetic background, the species and strain of the *Eimeria* and the infection history of the host (Rose and Long, 1969). All species of *Eimeria* are immunogenic; however some are more immunogenic than others. *Eimeria maxima*, *E. brunette* and *E. praecox* are considered the most immunogenic when compared to *E. tenella* and *E. maxima* (Williams, 2002). Natural killer cells, macrophages, and antigen specific T-and B-cells have all been demonstrated to play an active role in *Eimeria* infections (Lillehoj and Bacon, 1991). *Eimeria* infection caused an increase in the splenic and intestinal intraepithelial natural killer cell activity during the early phase of the secondary infection (Lillehoj and Bacon, 1991). Because of maternal and innate immunity and host genetics, the more resistant host will mount a more rapid response to infection when compared to

susceptible hosts (Shirley et al., 2005). Following infection with *Eimeria*, parasite-specific antibodies are present in the systemic circulation and mucosal secretions (Lillehoj and Trout, 1996). However, bursectomy did not affect the development of protective immunity against *Eimeria*, suggesting that the cell-mediated immune response is the primary means of disease response and that the humoral immune response may play a relatively minor role (Rose and Long, 1969). Several studies have evaluated the role of T-cells in the development of protection against *Eimeria* infection in chickens by damaging T-cell clonal expansion production and/or function. These studies evaluated thymectomy, cyclosporine A, betamethasone, dexamethasone and monoclonal antibodies against T-cell receptors (Lillehoj et al., 2004). The results showed that impaired T-cell function resulted in a decreased protective immune response against *Eimeria* infection. Peripheral blood lymphocytes and spleen cells from *E. maxima*-immune chickens protected naïve hosts against a live parasite challenge (Rose and Hesketh, 1982). In chickens immune to *E. tenella*, an increase in leukocytes in the lamina propria and ceca was observed when compared to naïve chickens, following an *Eimeria* challenge (Vervelde et al., 1996). During an *E. acervulina* infection, the ratio of heterophils to lymphocytes was increased in female Hubbard chicks (McFarlane and Curtis, 1989).

In addition to leukocyte activity during and *Eimeria* infection, cytokines have also been studied to determine their role in immunomodulation. Supernatants that contained gamma interferon from a culture of concanavalin-A stimulated spleen cells inhibited the intracellular development of *E. tenella* in the cells (Lillehoj, 1989). The

expression of gamma interferon was also shown to increase in the cecum and jejunum of white Leghorn chickens after infection with *E. tenella* and *E. maxima* when compared to control birds (Laurent et al., 2001). The response of IL 2 has also been evaluated following infection with *Eimeria* parasites. Primary and secondary infections with *E. acervulina* caused a significant increase in IL 2 genes in the spleen and intestine in chickens (Choi and Lillehoj, 2000).

Coccidiosis Control

The primary control for *Eimeria* populations within a poultry rearing facility is proper house management (Williams, 1998). However, because *Eimeria* oocysts are ubiquitous in the environment it is difficult to prevent birds from encountering oocysts (Shirley et al., 2005). Some common practices in the U.S. that may decrease the prevalence of these parasites includes removing caked litter and allowing the houses to air out for 2-3 weeks and subsequently topdressing with fresh litter before flock placement (Danforth, 1998). Many producers in Canada and European countries practice complete removal of litter. One of the most common methods for coccidiosis control includes the use of prophylactic drugs, classified as chemicals or ionophores which inhibit asexual stages of the *Eimeria* life cycle (Williams, 1998). The use of drugs to control coccidiosis in poultry was first introduced in 1936 with the discovery of sulfonamides (McDougald, 1998). When using prophylactic drugs a shuttle program is implemented to minimize resistance to the anticoccidials or they combine the two drug types during a flock grow out (Williams, 1999). Some of the more common drugs approved for use today include monensin, salinomycin, narasin, lasalocid, madauricin,

clopidol, robenidine, nicarbazin, amprolium, halofuginone and sulfonamides (Chapman, 1993). Monensin, narasin, salinomycin, lasalocid, clopidol and quinolines are more effective on day 1 of the life cycle; robenidine has the greatest activity on day 2 of the life cycle; nicarbazin, amprolium, halofuginone and sulfonamides have optimal effectiveness on d 3 of the life cycle (Reid, 1976). Although the drugs have been demonstrated to be effective, recent concerns of drug resistance and consumer concerns for drug use in poultry has stimulated an increased interest in the use of live vaccines for coccidiosis control (Williams, 2002).

Live vaccines have been successfully used in the commercial poultry industry for over fifty years (Allen and Fetterer, 2002). Their mode of action is attributed to recycling of initially low doses of oocysts which facilitates immunity (William, 1998). Edgar (1964) developed a planned immunization strategy where he administered small numbers of 8 different species in the feed or water during the first week of production. It has been demonstrated that daily dosages of 1 to 5 oocysts over a period of 20 d produced stronger immunity to *E. maxima*, *E. acervulina* and *E. tenella* (Joyner and Norton, 1973). The recycling of infection is a key factor to the development of protective immunity based on research demonstrating that birds reared in cages remained susceptible to an *Eimeria* challenge when compared to birds reared on litter (Chapman and Cherry, 1997). Additionally, multiple mild reinfections are more effective in developing immunity when compared to a single high dose of oocysts (Joyner and Norton, 1973). Some factors that may affect the build up of immunity include stressors or presence of other pathogens (Williams, 2002). Stressful events will cause bird

corticosterone levels to increase which stimulates higher primary cellular resistance and higher cell mediated immunity sensitization to coccidiosis (Gross, 1985).

Live vaccines include either attenuated or virulent *Eimeria* strains (Williams, 2002). Non-attenuated vaccines (Coccivac, Immunocox, Nobilis, COX ATM, and VAC- M) contain parasites that have been derived from laboratory or field strains (Allen and Fetterer, 2002). Attenuated vaccines (Livacox and Paracox) contain *Eimeria* species that have been treated to reduce their virulence (Davis, 1981). With this vaccine type, the infectious agents' pathogenicity is reduced without compromising its ability to elicit an immune response and this is achieved by passing the parasite through embryonated hens' eggs or by selecting an early stage of development (Shirley et al, 2005). All vaccines are administered during the first week post-hatch and should induce immunity (Danforth, 1998). Attenuated strains of vaccines have an advantage when compared to the non-attenuated form because they have low reproductive potentials which prevent tissue damage (Williams, 1999). Additionally, weight loss and high rates of mortality may result from possible infections caused by live vaccination (Shirley and Bellati, 1988). Another risk of using live vaccines is that it may introduce novel species of *Eimeria* that were not initially present in the poultry rearing environment (Chapman et al., 2002). The composition of most live vaccines used in broiler operations include live *E. acervulina*, *E. maxima*, and *E. tenella* oocysts due to their high levels of pathogenicity (Shirley et al., 2005). Because laying birds have a longer production cycle, all seven species may be included in their vaccines (McDougald et al., 1990). When selecting a vaccination program, however, other factors should be considered such as the cost of

producing the oocysts, the epidemiology of the *Eimeria* species in different rearing facilities (Williams, 2002).

The efficacy of a vaccine is influenced heavily on the method of administration and inadequate administration may lead to ineffective protection from disease (Chapman and Cherry, 1997). Vaccination delivery methods include intra-ocular, hatchery spray, edible gel, spray-on-feed, intra-yolk sac and *in-ovo* administration (Williams, 2002). Intra-ocular administration is achieved by the vaccine being sprayed into the eye, allowing the oocysts to pass down the nasalachrymal duct to the intestine by way of the buccal cavity (Chapman and Cherry, 1997). The hatchery spray method sprays the vaccine over trays of chicks, which facilitate oral by self preening and pecking drops of their neighbors (Bafundo and Jeffers, 1990). Edible gel administration involves incorporating the vaccine in an edible gel that's placed in chick trays at the hatchery or in the poultry house immediately after placement (Danforth, 1998). The *in-ovo* administration method would deliver a vaccine dose into the allantoic cavity of a 10-d old embryo Thaxton and Thaxton, 2004). Intra-yolk administration uses a needle to deliver live vaccines in yolk sac on day 18 of incubation (Thaxton and Thaxton, 2004). The efficiency of administration should be as high as possible to avoid wasted product and to ensure successful administration to individual bird (Chapman, 1997).

Probiotics

As the concern for drug use and live vaccination in commercial poultry programs continue, the need for alternative strategies should be developed. Recent research presents evidence that various dietary (Lee et al., 2009); and probiotic supplements

(Stringfellow et al, 2011) can influence host immunity against *Eimeria*. Probiotics are viable nonpathogenic bacteria that once consumed by the host, induce beneficial effects on the gut microflora (Fuller, 2004). The chicken intestinal tract is colonized by bacteria soon after hatching (Brisbin et al., 2008). The dynamics of this microbiota constantly changes over the course of the life of the host (Brisbin et al., 2008). In addition to probiotics, many factors influence the establishment of the microbiota including age and the presence of antibiotics or drugs from the diet (Brisbin et al., 2008).

Successful probiotic administration in poultry was first demonstrated in 1973 by Nurmi and Rantala. They reported that newly hatched chicks could be protected against *Salmonella* colonization of the gut by dosing them with a suspension of gut contents that was prepared from healthy adult chickens. Subsequent studies showed that this method can also offer protection against *Clostridium perfringens*, *Clostridium botulinum* and *Yersinia enterocolitica* (Fuller, 1989). The beneficial effects of probiotic bacteria have been well documented and include: competitive exclusion (Nurmi and Rantala, 1973; Soerjadi et al., 1982), enhanced intestinal development and integrity (Savage et al., 1981; Williams, 1998) and increased or modified resident microbial communities (Fuller, 1989; Hosoi et al., 1999; Jin et al., 2000). The more common probiotic preparations include *Lactobacillus*, *Bifidobacterium*, *Candida*, *Streptococcus*, *Enterococcus*, *Bacillus* and *Aspergillus* species (Fuller, 1989). Probiotic supplementation in poultry diets have been shown to enhance gut health and defense mechanisms, as well as both innate and adaptive immunity (Koenen et al., 2004; La Ragione et al., 2004; Farnell et al., 2006). The use of probiotics in animal diets has also

been shown to be effective against several enteric pathogens negatively affecting poultry production including *Escherichia coli* (La Ragione et al., 2004), *Salmonella* spp. (La Ragione et al., 2004) and *Eimeria* spp. (Dalloul et al., 2003). Enhancing the gut microflora may prove to be effective in potentiating the host immune response, subsequently improving the effects of coccidiosis vaccines in poultry (Dalloul et al., 2005). Lee and colleagues (2007) evaluated the influence of a commercial *Pediococcus*-based probiotic on coccidiosis in broiler chicks. They found that a 0.1% supplementation of the probiotic improved weight gain when compared to broilers that did not receive probiotic. Another study revealed that broilers fed *Lactobacillus* based probiotics had significantly higher gamma interferon and IL levels in the sera and intestinal secretions following an *Eimeria acervulina* challenge (Dalloul et al., 2005). Farnell and coworkers (2006) evaluated the upregulation of oxidative burst and degranulation in heterophils stimulated with probiotic bacteria. They found that of the 11 isolates evaluated, *Bacillus subtilis*, *Lactococcus lactis lactis*, and *Lactobacillus acidophilus* caused a higher heterophil response *in vitro*. A similar response was observed when the isolates were administered to the broiler chicks. Dalloul and colleagues (2003) evaluated the effect of feeding a *Lactobacillus*-based probiotic to broilers to determine its effect on intestinal intraepithelial lymphocyte subpopulations and subsequent protection against coccidiosis.

These researchers found that probiotic administration caused an increase in the expression of CD3, CD4, CD8 and $\alpha\beta$ TCR when compared to the control birds. Additionally, a reduction in *Eimeria* oocysts was observed in the broilers being fed the

probiotic diet. Results from this study indicate that the probiotic diet has a positive impact on the immune response during a coccidiosis infection. Stringfellow et al., (2011) evaluated the effect of probiotic administration on oxidative burst of broiler heterophil and monocyte and proliferation of T lymphocytes following a coccidiosis vaccination; their findings showed that probiotic administration increased all evaluated parameters, at multiple time points throughout the study (d 7, 14 or 21).

Avian Beta-defensins

Another integral part of both innate and acquired immunity is the activity of defensins, which are cationic antimicrobial peptides that play a vital role in the epithelial defense barrier (Sugiarto and Yu, 2006). Defensins are divided into three groups: α , β and θ (Abdel-Mageed et al., 2008). Avian β -defensins have been isolated and identified in the blood, epithelial cells, lungs, bone marrow, tongue, trachea, intestine and the bursa of Fabricius in chickens and turkeys (Zhao et al., 2001; Sugiarto and Yu, 2006). There have been 14 AVBD genes identified so far (Lynn et al., 2007). Avian β -defensins have been found in the tongue, esophagus, crop, proventriculus, gizzard, small intestine, large intestine, caeca, colon, cloaca, pancreas, liver, gall bladder, trachea, lung, air sacs, kidneys, testes, vas deferens, ovary, oviduct, infundibulum, uterus, vagina, egg yolk, skin, thymus, spleen, bursa, heart, skeletal muscle, brain, bone marrow and leukocytes (Dijk et al., 2008), with each tissue having a varying degrees of gene expression.

Defensins are cationic molecules have a broad spectrum of antimicrobial activity which includes bacteria, protozoal, some fungi and enveloped virus (Donovan and Topley, 2003). There are limited studies analyzing the antimicrobial effects of AVBD.

The exact effector mechanism of AVBD is not fully understood, however, it has been speculated that the actions are based on its cationic and amphipathic features (Sugiarto and Yu, 2006). A hypothetical carpet-wormhole model has been described for their mechanism of action in inhibiting and/or killing microorganisms (Ganz, 2003). In this model, the cationic peptides of the defensin structure are able to electrostatically interact with negatively charged components (LPS, lipoteichoic acid and anionic phospholipids) passing the membrane via the self-promoted uptake pathway which is the destabilization of the outer membrane perturbing its otherwise selective properties (Hancock, 1997). This interaction is followed by: 1. accumulation of peptides parallel to the membrane and formation of transient pores in the membrane; 2. Intracellular interactions with DNA, RNA or proteins may disable protein synthesis and function; and 3. Aggregate channels may be formed (Hancock, 1997).

Milona and colleagues (2007) evaluated the antimicrobial activity of the AVBD genes in the intestine of chickens and determined that the genes act as antimicrobial agents that constitute an integral part of the avian host innate defense system. The gene expression of AVBD has been shown to increase in response to antigens such as LPS, a Gram-negative bacteria cell wall component (Yoshimura et al., 2006; Abdel-Mageed et al., 2008). Recently, Abdel-Mageed et al., (2008) determined that several AVBD genes were significantly increased in the vagina of hens in response to LPS treatment. It is possible that gene expression of AVBD may also increase in response to a coccidial infection. Evans and colleagues (1995) evaluated the antimicrobial activity of chicken AVBD 1 and 2 and turkey AVBD 1 and 3 against a panel of selected avian pathogens

and human pathogens. The defensins used in their study were collected from 6-week-old broiler and turkey poult heterophils. Their findings showed that at a concentration of 2-16 $\mu\text{g/mL}$, all four defensins caused a greater than 90% reduction in the survival of *Candida albicans*, *Salmonella* Enteritidis, and *Campylobacter jejuni*. However, none were able to reduce the survival of *Pasteurella multocida* by 90% at the maximum concentration. All of the defensins tested, with the exception of turkey AVBD 3, were able to achieve a 90% reduction in the survival of *Bordetella avium*, *Escherichia coli* and *Salmonella* Typhimurium at the 16 $\mu\text{g/mL}$ concentration. Defensins isolated from avian heterophils have also been demonstrated to have antimicrobial activity against *Mycoplasma gallisepticum* (Sugiarto and Yu, 2006). Higgs and colleagues (2005) discovered AVBD 11 and 12 and subsequently determined the ability of AVBD 11 to kill *Salmonella* Typhimurium, *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus pyogenes*. They found that AVBD 11 was most effective against all of the test organisms except for *Staphylococcus aureus*. At concentrations of 500 $\mu\text{g/mL}$, AVBD 11 was able to completely kill *Listeria monocytogenes* and *Salmonella* Typhimurium at a concentration of 250 $\mu\text{g/mL}$. At the higher concentration, AVBD 11 only caused partial killing of *Escherichia coli* and *Streptococcus pyogenes*. Avian beta defensin 11 was shown to be highly expressed in the small intestine, liver, gall bladder and the spleen. They suggested that due to the high expression of AVBD 11 in the intestine and its ability to kill the pathogens tested, the defensin plays a key role in intestinal bacteria. Conversely, AVBD 12 was found to be expressed in the liver and gall bladder and may not be effective in killing the pathogens tested. Dijk and

coworkers (2007) evaluated the expression of AVBD 6 in the chicken digestive tract and its antimicrobial activity against food-borne pathogens. Using real time PCR, the researchers reported that the expression of AVBD 6 was highly expressed in the esophagus and crop, with moderate expression in the glandular stomach and low expression throughout the intestinal tract. Antimicrobial activity was tested against *Bacillus cereus*, *Campylobacter jejuni*, *Candida albicans*, *Clostridium perfringens*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, *Salmonella Typhimurium*, *Staphylococcus aureus*, and *Streptococcus pyogenes*. Antimicrobial activity was demonstrated against all test organisms. Following transmission electron microscopic analysis of the *Clostridium perfringens* cells, they reported that there was a dose-dependent morphological effect. A 30-min treatment of 10^8 CFU/ml *Clostridium perfringens* cells with AVBD 6 concentrations ranging from 1.56 to 25 μ g/ml induced changes in a dose dependent manner. Visualization revealed clumping of intracellular material and irregular septum formation during cell division at lower concentrations. Higher concentrations resulted in cytoplasm retraction and detachment of the cytoplasmic membrane from the peptidoglycan layer.

Conclusion

The purpose of the current research was to evaluate the immunomodulatory effects of probiotic administration in broilers vaccinated against coccidiosis or receiving an ionophore. Experiments were conducted to measure differences in heterophil/monocyte oxidative burst levels, lymphocyte proliferation and defensin gene expression. The hypothesis of this study was that probiotic administration will have a

positive effect on either mucosal immunity or vaccine responsiveness facilitating an improvement in vaccine efficacy during a broiler grow-out trial.

CHAPTER III

EVALUATION OF PROBIOTIC ADMINISTRATION ON THE IMMUNE RESPONSE OF COCCIDIOSIS VACCINATED BROILERS

Introduction

Avian coccidiosis is an enteric disease of birds caused by protozoan parasites of the genus *Eimeria* (Chapman et al., 2005). Economic losses are incurred during commercial rearing of poultry due to increased mortality, losses in weight gain and increased feed conversion ratios (Williams, 1999). The estimated worldwide annual economic loss incurred by the U.S. poultry industry due to coccidiosis and its control measures is \$3 billion (Dalloul and Lillehoj, 2006). The route of *Eimeria* infection is via the consumption of fecal droppings of infected birds (Li et al., 2005). Due to the high cost of bedding material, its limited availability and the cost of litter disposal, broiler producers routinely recycle or top-dress litter for subsequent flocks (Malone et al., 1992). Unfortunately, the pathogen persists in the litter, infecting subsequent flocks. Coccidiostats have been used to control this disease, however, *Eimeria* species have developed resistance to both chemical and ionophore-type prophylactic drugs (Chapman, 1997). Due to drug resistance by *Eimeria* species and consumer demand for drug-free poultry, there has been increased interest in the use of live vaccines for disease control (Chapman, 1997; Li et al., 2005).

Vaccination induces protective immunity by exposing the chicken's immune system to *Eimeria* antigens, so that it may better respond to field strain *Eimeria* infection

(Chapman et al., 2005). One disadvantage of using live vaccines is that they are typically administered to chicks during the first 1-7 d of its life, resulting in a low-level infection (Li et al., 2005). Vaccination during this period increases the risk of disease because it requires at least 7 to 10 d, following vaccination, for the stimulation of the acquired immune response (Kogut et al., 1998). An infection at this stage of a hatchling's life may cause an early reduction in growth and increase the chick's susceptibility to secondary infections resulting in diseases such as necrotic enteritis (Chapman et al., 2002; Williams, 2005). Maintenance of a healthy intestinal tract and normal microflora are key factors to consider that may improve *Eimeria*-vaccines in the first week post-vaccination (Dalloul and Lillehoj, 2005).

The beneficial effects of probiotic bacteria have been well documented and include: competitive exclusion (Nurmi and Rantala, 1973; Soerjadi et al., 1982), enhanced intestinal development and integrity (Savage et al., 1981; Williams, 1998) and increased or modified resident microbial communities (Fuller, 1989; Hosoi et al., 1999; Jin et al., 2000). Recent evidence suggests that probiotics may enhance host defenses and improve vaccine response because of the influence of bacteria on host immunity and intestinal integrity against enteric parasites (Dalloul et al., 2003; Farnell et al., 2003; Koenen et al., 2004). The authors found limited research focusing on the immunopotentiating effects of probiotic bacteria in coccidiosis-vaccinated broilers. We evaluated oxidative burst (innate immunity) of chicken heterophils and monocytes and the proliferation of lymphocytes (adaptive immunity) to investigate the effects of a commercial probiotic in vaccinated broilers.

Materials and Methods

Birds and Treatments

A total of 400 Cobb 500 male broilers were obtained from a local commercial hatchery. Treatments consisted of a negative control, probiotic, vaccine, and a probiotic + vaccine combination group. One hundred chicks were placed in each pen on fresh litter (1 pen per treatment), achieving a final rearing density of 0.8 sq ft/bird. Chicks were provided water, age appropriate supplemental heat as needed and fed an industry-based diet formulated from current industry nutrient specifications. The nutrient profiles of the two diets were identical to previous trials conducted in our laboratory (Table 1) (Lee et al., 2009). These studies were conducted according to approved guidelines of the Texas A&M University Institutional Animal Care and Use Committee.

Experimental Design

A 2 X 2 ANOVA based experiment was conducted to evaluate the effect of probiotic administration and its ability to modulate immune function in broilers to coccidiosis vaccination. On d 7, 14 and 21, heterophils and mononuclear leukocytes from the peripheral blood of 10 broilers were collected and combined into four pooled (3-4 birds per sample) samples for each group. To evaluate innate immunity, an oxidative burst assay was conducted on heterophils and monocytes using a fluorescent plate reader and an indicator of reactive oxygen species. Adaptive immunity was measured with a lymphocyte proliferation assay, which measured the ability of lymphocytes placed in short term tissue culture to undergo a clonal proliferation when stimulated *in vitro* by a mitogen.

Table 1. Calculated nutrient concentrations of experimental diets fed to broilers

Calculated nutrient concentration, % unless noted	Study 1	Study 2
Protein	21.5	22.0
Methionine	0.58	0.59
TSAA	0.95	0.97
Lysine	1.22	1.27
Threonine	0.84	0.86
Arginine	1.44	1.47
Tryptophan	0.25	0.26
Calcium	1.00	1.00
Available phosphorus	0.51	0.51
Sodium	0.22	0.22
ME, kcal/kg	3,080	3,080

Probiotic Administration

The probiotic, Biomin® PoultryStar (Biomin GmbH, Herzogenburg, Austria), contained 2.3×10^{12} CFU per pound of lactic acid producing bacteria including: *Enterococcus faecium*, *Pediococcus acidilactici*, *Bifidobacterium animalis* and *Lactobacillus reuteri*. The probiotic was administered through the drinking water at a concentration of 20g/1000 birds/d which delivered 1×10^8 CFU/bird/d. Birds were administered probiotic from d 0-4, 11-13 and 15-17 per manufacturer's recommendations. Dose uniformity was not verified in these studies. The assumption was made that all birds drank similar amounts of water. The authors adjusted the dosage of the probiotic according to the commercial weekly water consumption rates of broilers (North and Bell, 1990).

Vaccination

Birds in the vaccinated groups received Coccivac®-B (Intervet/Schering - Plough Animal Health, Summit, N J) using a commercial (Spraycox® II) spray cabinet on day of hatch. Chicks were allowed to preen under elevated light intensity for at least one hour prior to placement.

Leukocyte Isolation

Peripheral blood was collected by decapitation or venipuncture, and EDTA (Sigma-Aldrich, St. Louis, MO) was used as an anticoagulant. Blood was pooled from 10 birds per treatment (n= 4 separate pools/gradients per treatment). The polymorphonuclear and mononuclear cell fractions were isolated as previously described (Kogut et al., 1995). Briefly, blood was mixed with 1 % methycellulose (Sigma-

Aldrich), dissolved in Roswell Park Memorial Institute (**RPMI**)-1640 media (Mediatech Inc., Herndon, VA) at a ratio of 1:1.5 and centrifuged at 37 x g for 15 min. The supernatant was removed and re-suspended in Hanks balanced salt solution without calcium or magnesium (Mediatech Inc.) at a ratio of 1:1. The suspension was layered over a 1.077/1.119 Histopaque (Sigma-Aldrich) discontinuous gradient and centrifuged at 235 x g for 60 minutes. The suspension above the 1.077 Histopaque was collected for the mononuclear cell fraction and the interface of the two gradients was collected for polymorphonuclear cells. Cell types were then separated into conical tubes and washed with 40 mL of RPMI-1640 by centrifugation at 603 x g for 15 min. Following the RPMI-1640 wash, a complete solution of RPMI-1640 was added to reconstitute the cells. The media consisted of 5% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA); 4 mM L-glutamine, 10,000 U penicillin/mL, 10 mg streptomycin/mL (JR Scientific Inc., Woodland, CA); 1mM sodium pyruvate (Mediatech Inc.) and 0.1 mM non essential amino acid (MP Biomedicals, LLC, Solon, OH). All agonists and substrates were suspended in this complete RPMI-1640. The cells were then quantified using a Neubauer hemacytometer; the stock was adjusted to a working concentration of 1×10^7 cells/mL and stored on ice until used. Cell viability was determined by a commercially prepared trypan blue solution (Sigma-Aldrich). Heterophil preparation obtained by this method is typically > 95% pure and > 95% viable (Kogut et al., 1995). Although the authors significantly enriched for lymphocytes, a pure culture of these cells were not obtained. However the proliferation assay described below measures the expansion of viable cell numbers that results in an

increase in the activity of the mitochondrial dehydrogenases in the sample. Monocytes are not stimulated by the mitogen (ConA) used in the assay, therefore only the lymphocytes in the culture would proliferate and produce a significant signal.

Oxidative Burst Assay

Oxidative burst activity of heterophils and monocytes was measured using a Wallac fluorescent plate reader (Perkin Elmer, Boston, MA) and an indicator of reactive oxygen species, 2,7-dichlorofluorescein (**DCF-DA**; 0.2 mg/mL, Sigma-Aldrich) as previously described (Xie et al., 2002). A minimal amount (90 μ L) of ethanol was used to dissolve the DCF-DA. Cells (1 mL) were preincubated for 30 minutes with 125 μ L of phorbol-12-myristate-13-acetate (**PMA**; 20 μ g/mL of cells, Calbiochem, La Jolla, CA) at 42°C in a water-jacketed 5% CO₂ incubator. An equivalent volume of complete RPMI-1640 was added for the negative control treatments. Immediately after the preincubation period, 125 μ L of DCF-DA was added and samples were mixed and aliquoted (8 replicates per sample) into a clear 96-well flat-bottomed plate. Oxidative burst was then measured at an excitation/emission wavelength of 485/530 nm.

Mononuclear Cell Proliferation Assay

A modified cell proliferation assay (Pauly et al., 1973) was conducted to evaluate cell mediated immunity. Concanavalin A (**Con A**; Sigma-Aldrich; 20 μ g/mL) was used as a mitogen and water-soluble tetrazolium salt (**WST-1**; Clontech, Palo Alto, CA) was used as a colorimetric substrate to analyze cell proliferation. Briefly, 50 μ L of the mononuclear cell fraction was mixed with 50 μ L of Con A and 10 μ L of WST-1 inside a clear, 96-well, flat bottomed plate for 24 hours at 42°C in a water-jacketed 5% CO₂

incubator. An equivalent volume of complete RPMI-1640 was added in replacement of the Con A for the negative control treatments. The color change was measured at 450 nm by a colorimetric plate reader (Sunrise, Tecan, Austria).

Statistical Analysis

Statistical analysis was completed with the SPSS statistical software package (Chicago, IL). Data in all experiments were analyzed via a one-way ANOVA using the GLM procedure due to interactions between probiotic administration and coccidiosis vaccination, which were observed within all assays at multiple time points. Differences were deemed significant at $p \leq 0.05$ and means were separated using Duncan's multiple range test. Pooled blood from the chickens of each treatment served as the experimental unit for these studies.

Results and Discussion

The present investigation evaluated the effects of a commercially available probiotic on mediating the innate and adaptive immune response in broilers vaccinated with a commercially available live oocyst coccidiosis vaccine. Probiotic administration did not have an effect on the immune responses of vaccinated broilers on d 7 in either of the two studies. On d 7, the lymphocytes from all three treatment groups were found to have higher ($p \leq 0.05$) proliferation when compared with the negative control group in study 1 (Fig. 3.1). As shown in figures trial 1, vaccinated broilers treated with probiotic had significantly higher immune responses on d 14 in relation to other treatments. Oxidative burst of the heterophils in the probiotic group was also greater ($p \leq 0.05$) on d

14 when compared to the control group (Fig. 3.1). Similar results were also

Figure 3.1.

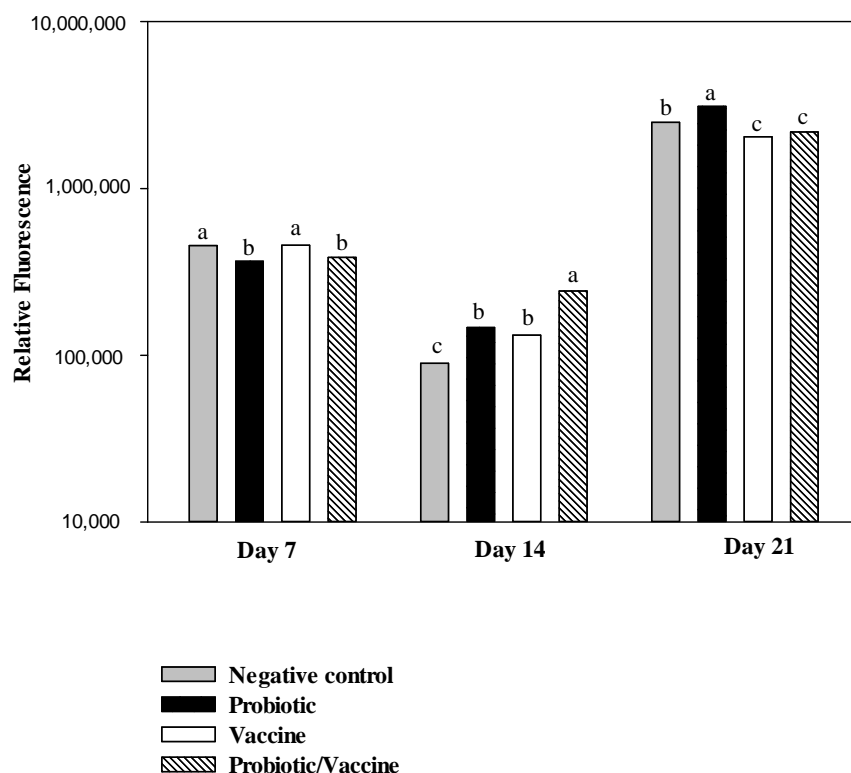


Figure 3.1. Oxidative burst of phorbol-12-myristate-13-acetate stimulated heterophils isolated from broilers fed an industry-based diet in combination with probiotic administration (water), coccidiosis vaccine administration, or a probiotic + vaccine on 7, 14 and 21 d of age. Each bar represents the mean (n = 4). Means within each d with different letters differ significantly ($p \leq 0.05$).

observed on d 14 in lymphocyte proliferation with the vaccine group (Fig. 3.3). No increases in immune responses were observed among any of the treatment groups when compared to the control group on d 21, except for the heterophil oxidative burst in the

probiotic treatment (Fig. 3.1-3.3). Limited differences in immune responses were observed in study 2 (Fig. 3.4-3.6). However, on d 7, heterophil and monocyte oxidative

Figure 3.2.

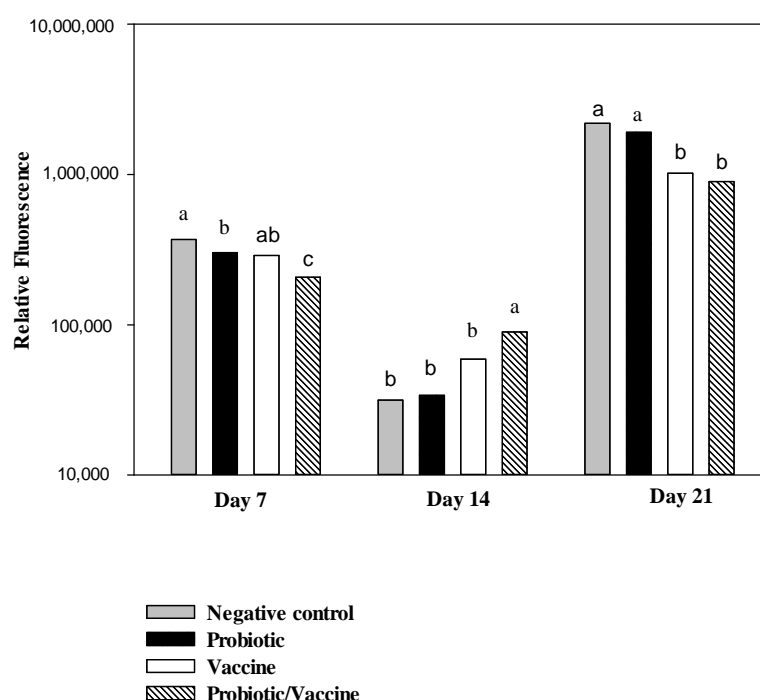


Figure 3.2. Oxidative burst of phorbol-12-myristate-13-acetate stimulated monocytes isolated from broilers fed an industry-based diet in combination with probiotic administration (water), coccidiosis vaccine administration, or a probiotic + vaccine on 7, 14 and 21 d of age. Each bar represents the mean (n = 4). Means within each d with different letters differ significantly ($p \leq 0.05$).

burst increased ($p \leq 0.05$) in the probiotic treatment and in the vaccine treatment respectively (Fig. 3.4-3.5). Higher ($p \leq 0.05$) levels in heterophil oxidative burst were also observed in the vaccine treatment group on d 21 (Fig. 3.1). The dietary crude protein concentration in study 1 was 21.5% and study 2 was 22.0% (Table. 1). In a previous study evaluating broilers vaccinated against coccidiosis, this 0.5% difference in

dietary crude protein attributed to an increase in body weight at d 14 of age (Lee et al., 2009). The authors wanted to further investigate whether this increase in protein affected the immune parameters evaluated in these studies.

Figure 3.3.

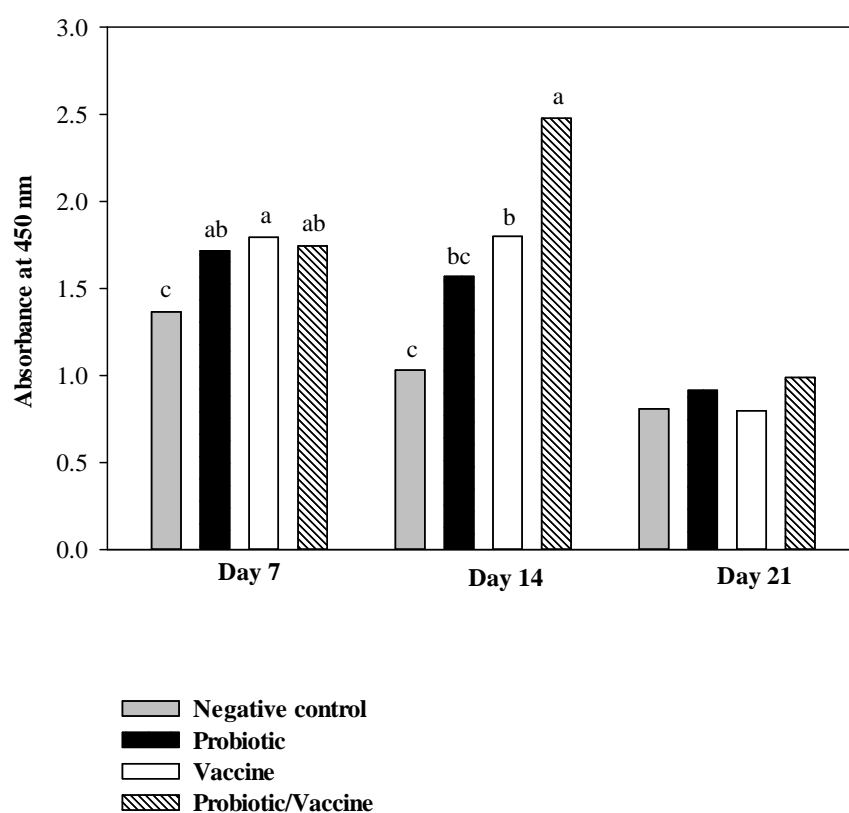


Figure 3.3. Proliferation of concanavalin A treated lymphocytes isolated from broilers fed an industry-based diet in combination with probiotic administration (water), coccidiosis vaccine administration, or a probiotic + vaccine on 7, 14 and 21 d of age. Each bar represents the mean (n = 4). Means within each d with different letters differ significantly ($p \leq 0.05$).

Figure 3.4.

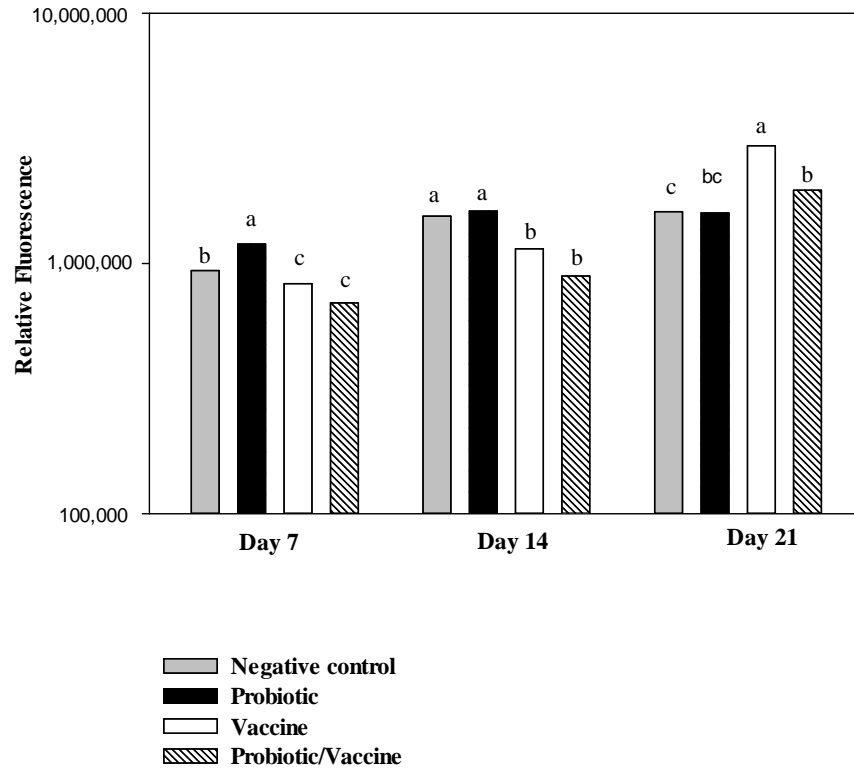


Figure 3.4. Oxidative burst of phorbol-12-myristate-13-acetate stimulated heterophils isolated from broilers fed an industry-based diet in combination with probiotic administration (water), coccidiosis vaccine administration, or a probiotic + vaccine on 7, 14 and 21 d of age. Each bar represents the mean (n = 4). Means within each d with different letters differ significantly ($p \leq 0.05$).

Figure 3.5.

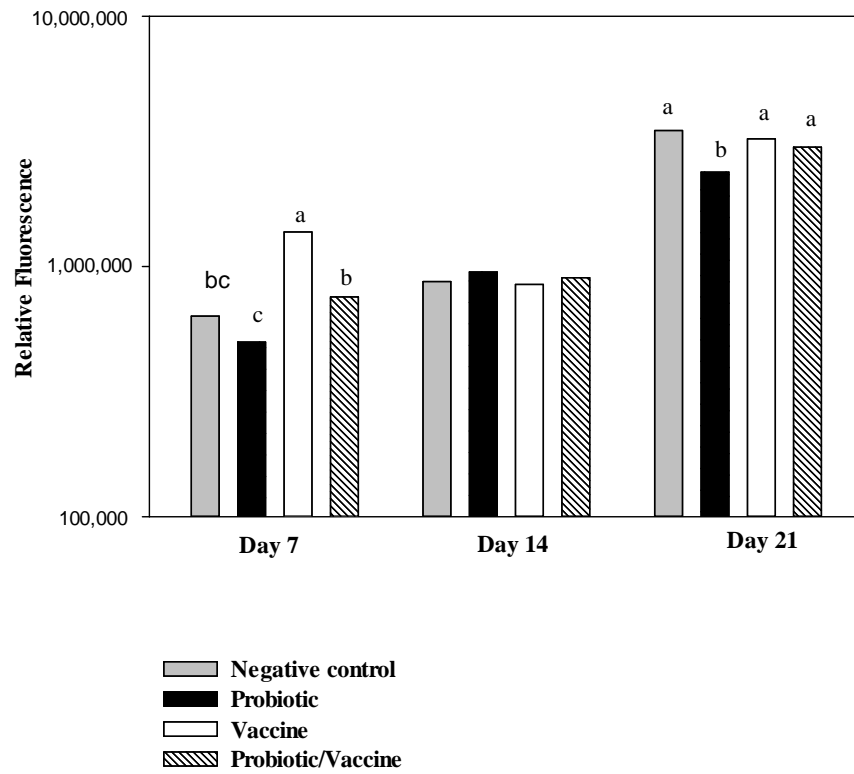


Figure 3.5. Oxidative burst of phorbol-12-myristate-13-acetate stimulated monocytes isolated from broilers fed an industry-based diet in combination with probiotic administration (water), coccidiosis vaccine administration, or a probiotic + vaccine on 7, 14 and 21 d of age. Each bar represents the mean (n = 4). Means within each d with different letters differ significantly ($p \leq 0.05$).

Figure 3.6.

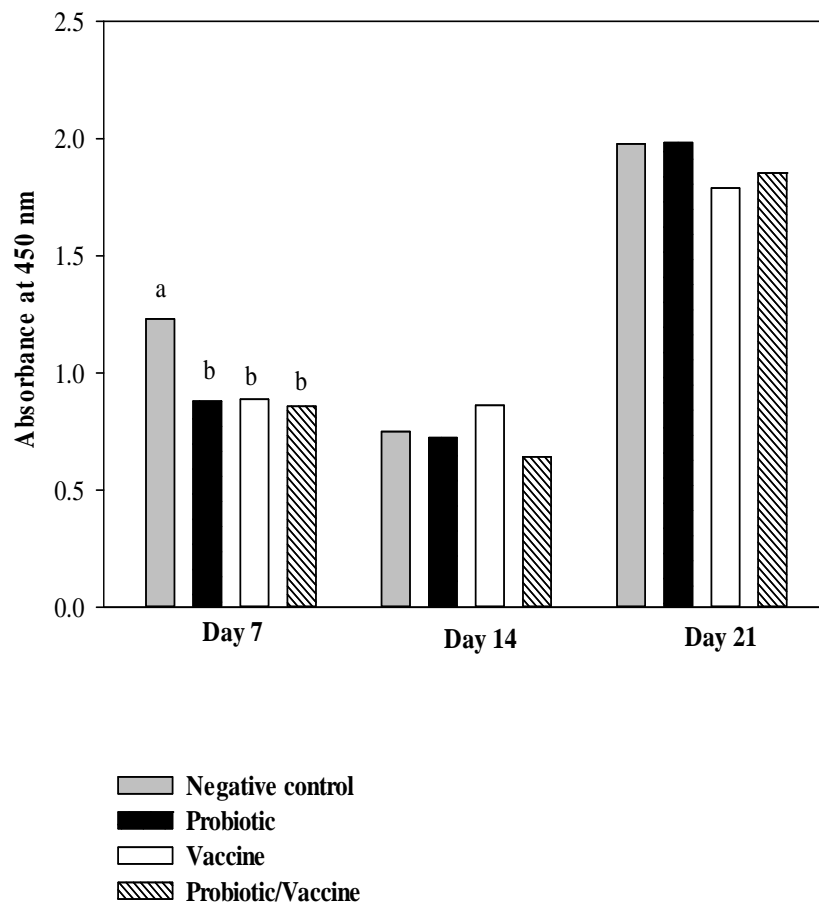


Figure 3.6. Proliferation of concanavalin A treated lymphocytes isolated from broilers fed an industry-based diet in combination with probiotic administration (water), coccidiosis vaccine administration, or a probiotic + vaccine on 7, 14 and 21 d of age. Each bar represents the mean (n = 4). Means within each d with different letters differ significantly ($p \leq 0.05$).

Probiotic bacteria activate the mucosal immune system through the stimulation of gut antigen-presenting cells to promote protection (Clancy, 2003). Additionally, Clancy (2003) concluded that the new term “immunobiotics” identifies bacteria that promote health through driving mucosal immune mechanisms. In the current study, the immunomodulatory effect of the probiotic and vaccine combination was best observed on d 14. The results showed that the addition of probiotic in the water of vaccinated broilers induced a significantly higher oxidative burst of monocytes and heterophils and a significantly higher lymphocyte proliferation when compared to the other groups. The exact mechanism mediating the increased immune response observed in this investigation is not fully understood because probiotics have an impact on a wide variety of host immune functions (Koenen et al, 2004; Nava et al., 2005; Donoghue et al., 2006). Similar results where probiotic bacteria administration modulated the host immune response to vaccines have been demonstrated in humans (De Vrese et al., 2005; Olivares et al., 2007) and pigs (Zhang et al., 2008).

Dalloul and colleagues (2003) demonstrated an increase in innate and adaptive responses against broilers infected with *Eimeria* and treated with a *Lactobacillus*-based probiotic; an increase in intra-epithelial lymphocytes expressing the surface markers CD3, CD4, CD8 and $\alpha\beta$ TCR and a reduction in oocyst shedding was observed in the birds who were being fed the probiotic. A study by Farnell and colleagues (2006) demonstrated that broiler chicks administered *Bacillus subtilis*, *Lactococcus lactis*, and *Lactobacillus acidophilus* had a higher heterophil response when compared to the negative controls, as indicated by improved oxidative burst and degranulation. The

present study confirms these findings, indicating that there is a relationship between improved immune function and probiotic administration.

These data suggest that probiotics have the potential to elevate immune cell activity in broilers who were vaccinated against coccidiosis. Probiotic administration may offer increased protection at the time of vaccination or have an adjuvant effect by modulating the host immune system. Future studies will be conducted to evaluate performance parameters and the effect of these treatments during an *Eimeria* challenge. Due to the recent issues associated with in-feed anticoccidial drugs and the high cost associated with coccidial infections, the need for improved vaccine programs for broilers is increasingly important.

CHAPTER IV

IMMUNOMODULATORY EFFECTS OF A COMMERCIALY AVAILABLE PROBIOTIC ON COCCIDIOSIS VACCINATION IN BROILERS

Introduction

Avian coccidiosis, commonly caused by the enteric parasite *Eimeria*, continues to have a negative economic impact on the U.S. poultry industry (Williams, 2003; Dalloul and Lillehoj, 2006). Over the past 50 years, the key control measure for reducing coccidiosis in the poultry industry has been the use of prophylactic drugs (Williams, 2005). In addition to prophylactic drugs, the use of live vaccines has been an increasingly important area of interest in broilers due to consumer concern of drug use in animal feed (Chapman et al., 2002; Williams, 2002). Coccidiosis vaccines have been traditionally used to protect breeder flocks, but their use in broiler flocks, while increasing in recent years, is still somewhat limited due to previous reports of reduced weight gain and increased feed conversion compared to prophylactically medicated broilers (Allen and Fetter, 2002; Williams, 2002). The production cycle of broilers is relatively short when compared to breeder flocks. As such, the ability of broilers to recover from any negative effects of vaccination and to initiate an appropriate immune response is relatively limited (Williams, 1998). It is important to concentrate on host nutrition and develop alternative strategies to improve the immune response and gut health following coccidiosis vaccination in broilers (Lee et al., 2011).

There are seven species of *Eimeria* that are pathogenic to chickens, with each having varying degrees of infectivity, therefore the capabilities of the host to defend against these pathogens rely on intestinal immunity (Allen and Fetterer, 2002). Enhancing the gut microflora may prove to be effective in potentiating the host immune response, subsequently improving the effects of anticoccidial vaccines in poultry (Dalloul et al., 2005). Probiotics are viable nonpathogenic bacteria that once ingested by the host, induce beneficial effects on the gut microflora (Fuller, 2004). Probiotic supplementation in poultry diets have been shown to enhance gut health and defense mechanisms, as well as both innate and adaptive immunity (Koenen et al., 2004; La Ragione et al., 2004; Farnell et al., 2006). The use of probiotics in animal diets has also been shown to be effective against several enteric pathogens negatively affecting poultry production including *Escherichia coli* (La Ragione et al., 2004), *Salmonella* spp. (La Ragione et al., 2004) and *Eimeria* spp. (Dalloul et al., 2003 a, b).

Concerns of drug-use have prompted the need to develop alternative or more cost-effective method to control pathogen prevalence (Williams, 2005). In two trials, we evaluated a commercial probiotic and its effect on the immune response of broilers vaccinated and subsequently challenged with *Eimeria* oocysts. Probiotics may stimulate host immune defense mechanisms or improve the efficacy of coccidiosis vaccines. Thus, we evaluated oxidative burst of heterophils and monocytes along with lymphocyte proliferation.

Materials and Methods

Birds and Treatments

In both trials, a total of 1,800 Cobb x Ross straight-run broilers were obtained from a local commercial hatchery. Treatments consisted of a negative control, probiotic, vaccine, and a probiotic + vaccine combination group. Forty-five chicks were placed in each pen (10 pens per treatment), achieving a final rearing density of 0.8 ft² (740 cm²) /bird with a 50:50 mixture of fresh and used litter in each pen. Birds were provided water, age appropriate supplemental heat (as needed), and fed a corn-soy diet formulated from current industry nutrient specifications. These studies were conducted according to approved guidelines of the Texas A&M University Institutional Animal Care and Use Committee. The feeding program for study one was as follows: starter phase (D 1-15), grower phase (D 16-30), finisher phase (D 31-40) and withdrawal phase (D 41-49). The feeding program for study two was as follows: starter phase (D 1-14), grower phase (D 15-28), finisher phase (D 29-35) and withdrawal phase (D 36-42).

Experimental Design

Two separate 2 X 2 factorial ANOVA designed experiments were conducted to evaluate the effect of probiotic administration and its ability to modulate immune function in broilers vaccinated against coccidiosis. In study 1, peripheral blood was collected on d 15, 30, 40 and 49. In the second study, peripheral blood was collected on d 14, 21, 28, 35 and 42. Following blood collection, heterophils and mononuclear leukocytes from 10 broilers were collected and combined into four pooled (3-4 birds per sample) samples for each treatment. To evaluate innate immunity, an oxidative burst

assay was conducted on heterophils and monocytes using a fluorescent plate reader and an indicator of reactive oxygen species. Adaptive immunity was measured with a lymphocyte proliferation assay, which measured the ability of lymphocytes placed in short term tissue culture to undergo a clonal proliferation when stimulated *in vitro* by a mitogen.

***Eimeria* infection (Trial 2 only)**

Field strain *Eimeria* oocysts from our laboratory (College Station, TX) consisting mainly of *Eimeria acervulina*, *E. maxima*, and *E. tenella* were used to challenge the birds in Trial 2. In addition to the 50/50 mix of fresh and used litter, to simulate a commercial rearing environment a direct application of 35,000 *Eimeria* oocysts were sprayed on the litter of all pens on d 14. To induce a clinical infection, all birds received an oral challenge of 550,000 oocysts on d 35.

Probiotic Administration

The probiotic, Biomin® PoultryStar (Biomin GmbH, Herzogenburg, Austria), contained 2.3×10^{12} cfu/kg of lactic acid producing bacteria including: *Enterococcus faecium*, *Pediococcus acidilactici*, *Bifidobacterium animalis* and *Lactobacillus reuteri*. The probiotic was administered through the drinking water at a concentration of 20 g/1000 birds per d which delivered 1×10^8 cfu/bird per d. Probiotic administration occurred on d 0-4, 10-12, 14-16, 29-31 and 39-41 per manufacturer's recommendations in study 1 and on d 0-3, 8-10, 13-15, 27-29 and 34-36. The assumption was made that all birds drank similar amounts of water. The authors

adjusted the dosage of the probiotic according to the commercial weekly water consumption rates of broilers (North and Bell, 1990).

Vaccination

Birds in vaccinated groups received Coccivac[®]-B (Intervet/Schering-Plough Animal Health, Summit, NJ) using a commercial spray cabinet (Spraycox[®] II; Intervet/Schering-Plough Animal Health) on d of hatch. Chicks were allowed to preen under elevated light intensity for at least one hour prior to placement.

Leukocyte Isolation

Peripheral blood was collected by decapitation (14 and 15 d of age only) or venipuncture, and EDTA (Sigma-Aldrich, St. Louis, MO) was used as an anticoagulant. Blood was pooled from 10 birds per treatment (n = 4 separate pools/gradients per treatment). The polymorphonuclear and mononuclear cell fractions were isolated as previously described (Kogut et al., 1995). Briefly, blood was mixed with 1 % methylcellulose (Sigma-Aldrich), dissolved in Roswell Park Memorial Institute (RPMI)-1640 media (Mediatech Inc., Herndon, VA) at a ratio of 1:1.5 and centrifuged at 37 x g for 15 min. The supernatant was removed and re-suspended in Hanks balanced salt solution without calcium or magnesium (Mediatech Inc.) at a ratio of 1:1. The suspension was layered over a 1.077/1.119 Histopaque (Sigma-Aldrich) discontinuous gradient and centrifuged at 235 x g for 60 minutes. The suspension above the 1.077 Histopaque was collected for the mononuclear cell fraction and the interface of the two gradients was collected for polymorphonuclear cells. Cell types were then separated into conical tubes and washed with 40 mL of RPMI-1640 by centrifugation at 603 x g for 15

min. Following the RPMI-1640 wash, a complete solution of RPMI-1640 was added to reconstitute the cells. The media consisted of 5% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA); 4 mM L-glutamine, 10,000 U penicillin/mL, 10 mg streptomycin/mL (JR Scientific Inc., Woodland, CA); 1mM sodium pyruvate (Mediatech Inc.) and 0.1 mM non essential amino acid (MP Biomedicals, LLC, Solon, OH). All agonists and substrates were suspended in this complete RPMI-1640. The cells were then quantified using a Neubauer hemacytometer (Electron Microscopy Sciences, Hatfield, PA); the stock was adjusted to a working concentration of 1×10^7 cells/mL and stored on ice until used. Cell viability was determined by a commercially prepared trypan blue solution (Sigma-Aldrich). Heterophil preparation obtained by this method was typically >95% pure and >95% viable (Kogut et al., 1995). Although the authors significantly enriched for lymphocytes, a pure culture of these cells were not obtained. The proliferation assay described below measured the clonal expansion of viable cell numbers which resulted in an increase in the activity of the mitochondrial dehydrogenases in the sample. Monocytes are non-proliferative cells; therefore, only the lymphocytes in the culture should have proliferated and produced a significant signal.

Oxidative Burst Assay

Oxidative burst of heterophils and monocytes was measured using a Wallac fluorescent plate reader (Perkin Elmer, Boston, MA) and an indicator of reactive oxygen species, 2,7-dichlorofluorescein (**DCF-DA**; Sigma-Aldrich) as previously described (Xie et al., 2002). A minimal amount (90 μ L) of ethanol was used to dissolve the DCF-DA. Cells (1 mL) were preincubated for 30 minutes with 125 μ L of phorbol-12-myristate-13-

acetate (**PMA**; 20 µg/mL, Calbiochem, La Jolla, CA) at 42°C in a water-jacketed 5% CO₂ incubator. An equivalent volume of complete RPMI-1640 was added for the negative control treatments. Immediately after the preincubation period, 125 µl of DCF-DA (0.2 mg/mL) was added and samples were mixed and aliquoted (8 replicates per sample) into a clear 96-well flat-bottomed plate. Oxidative burst was then measured at an excitation/emission wavelength of 485/530 nm.

Lymphocyte Proliferation Assay

A modified cell proliferation assay (Pauly et al., 1973) was conducted to evaluate cell mediated immunity. Concanavalin A (**Con A**; 20 µg/mL, Sigma-Aldrich) was used as a mitogen and (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (**MTT**; Sigma-Aldrich; 5mg/ mL) was used as a colorimetric substrate to analyze cell proliferation. Briefly, 75 µl of the mononuclear cell fraction was mixed with an equal volume of Con A, suspended in complete RPMI, inside a clear 96-well, flat bottomed plate for 24 hours at 42°C in a water-jacketed 5% CO₂ incubator. An equivalent volume of complete RPMI-1640 was added in replacement of the Con A for the negative control treatments. Following incubation, 15 µL of MTT was added to the cells and incubated for 4 hours at 42°C. Immediately after incubation 150 µL of MTT solvent (Sigma-Aldrich; 0.1 N HCL in anhydrous isopropanol) was added to the cells to dissolve the MTT formazan, resulting in a color change. The color change was measured at 450 nm by a colorimetric plate reader (Sunrise, Tecan, Austria).

Statistical Analysis

Statistical analysis was completed with the SPSS statistical software package (Chicago, IL). Data in all experiments were analyzed via a one-way ANOVA using the GLM procedure due to interactions between probiotic administration and coccidiosis vaccination, which were observed within all assays at multiple time points. Differences were deemed significant at $P \leq 0.05$ and means were separated using Duncan's multiple range test. Pooled blood from the chickens of each treatment served as the experimental unit for these studies.

Results

Experiment 1

Figure 4.1 shows the oxidative burst activity from PMA-stimulated heterophils. Probiotic + vaccine increased ($p \leq 0.05$) heterophil oxidative burst on d 15, when compared to the other treatments. On d 30, samples were not able to be obtained for the vaccine and the probiotic + vaccine treatment. From the samples that were obtained, the probiotic administration was not effective in increasing ($p \leq 0.05$) heterophil oxidative burst when compared to the negative controls. All treatments increased ($p \leq 0.05$) heterophil oxidative burst on d 40 and 49 when compared to the negative controls. The highest ($p \leq 0.05$) stimulation was observed with the vaccine group.

Figure 4.1.

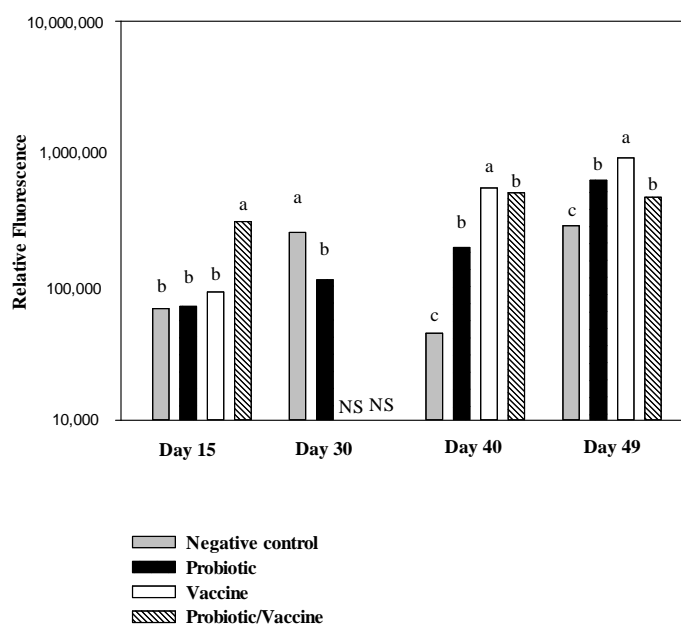


Figure 4.1. Oxidative burst of stimulated heterophils isolated from broilers fed an industry-based diet in combination with probiotic administration, coccidiosis vaccine administration, or a probiotic + vaccine on 15, 30, 40 and 49 d of age. Each bar represents the mean (n=4). No sample (NS) was obtained on d 30 in the probiotic + vaccine or vaccine treatment. Means within each d with different letters differ significantly ($p \leq 0.05$).

Figure 4.2 shows the oxidative burst activity from PMA-stimulated monocytes. Monocyte oxidative burst was elevated ($p \leq 0.05$) in all treatments on d 15 when compared to the negative controls. Probiotic administration elevated ($p \leq 0.05$) response when compared to the negative controls on d 30. On d 40 and 49 an increase ($p \leq 0.05$) in monocyte oxidative burst was observed in the vaccine group. The probiotic + vaccine treatment also increased on d 49 when compared to the negative controls.

Figure 4.2.

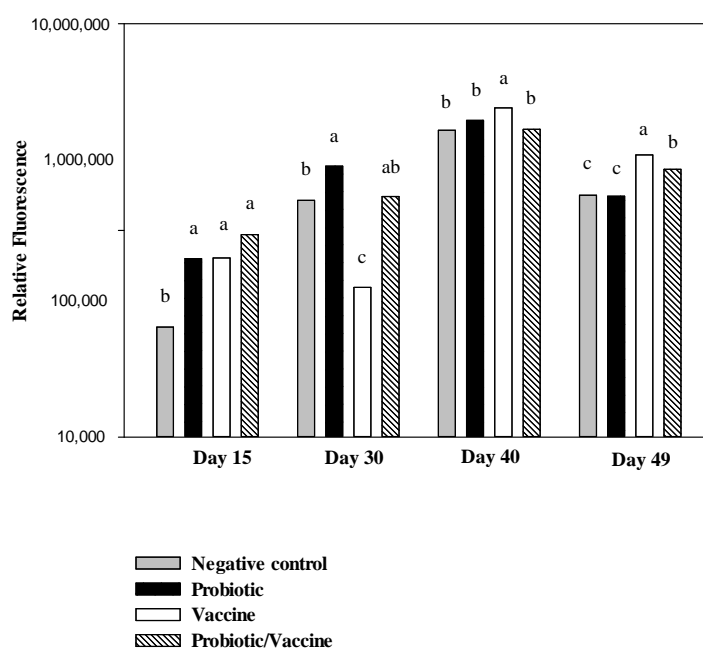


Figure 4.2. Oxidative burst of stimulated monocytes isolated from broilers fed an industry-based diet in combination with probiotic administration, coccidiosis vaccine administration, or a probiotic + vaccine on 15, 30, 40 and 49 d of age. Each bar represents the mean (n=4). Means within each d with different letters differ significantly ($p \leq 0.05$).

Clonal expansion of Con A-stimulated lymphocytes was measured using a colorimetric plate reader as shown in Figure 4.3. No increases ($p \leq 0.05$) were observed in lymphocyte proliferation among any of the treatments on d 15 when compared to the negative controls. However, differences ($p \leq 0.05$) were observed in the probiotic + vaccine treatment when compared to the probiotic and the vaccine group. On d 30, no significant differences were observed among any of the treatment groups. Lymphocyte proliferation increased ($P \leq 0.05$) in the vaccine treatment when compared to all other treatments on d 40. Lymphocyte proliferation was greater ($p \leq 0.05$) in the

probiotic and vaccine alone groups when compared to the negative controls, while the probiotic + vaccine group showed decreased ($p \leq 0.05$) proliferation.

Figure 4.3.

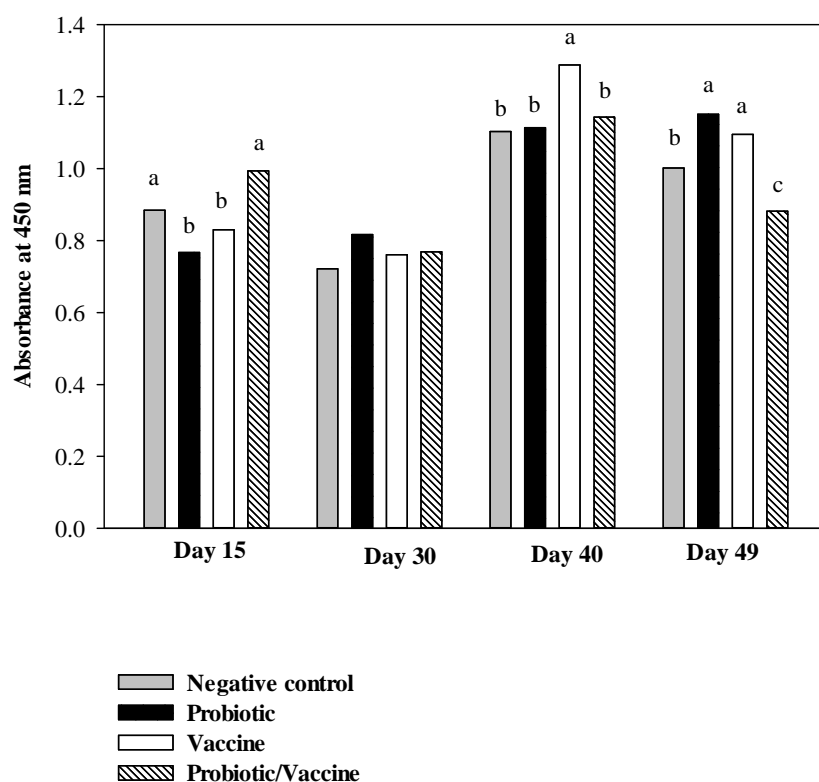


Figure 4.3. Proliferation of lymphocytes isolated from broilers fed an industry-based diet in combination with probiotic administration, coccidiosis vaccine administration, or a probiotic + vaccine on 15, 30, 40 and 49 d of age. Each bar represents the mean ($n=4$). Means within each d with different letters differ significantly ($p \leq 0.05$).

Experiment 2

Figure 4.4 shows the oxidative burst activity from PMA-stimulated heterophils.

On d 14 and 28, heterophil oxidative burst was greater ($p \leq 0.05$) in the probiotic +

vaccine broilers, relative to the negative controls. Heterophil oxidative burst was also higher ($p \leq 0.05$) in the probiotic alone and vaccine alone broilers on d 35 relative to the controls. On d 21 and 42, heterophil oxidative burst failed to increase ($p \leq 0.05$) in the probiotic + vaccine combination broilers as compared to the negative control and probiotic alone group.

Figure 4.4.

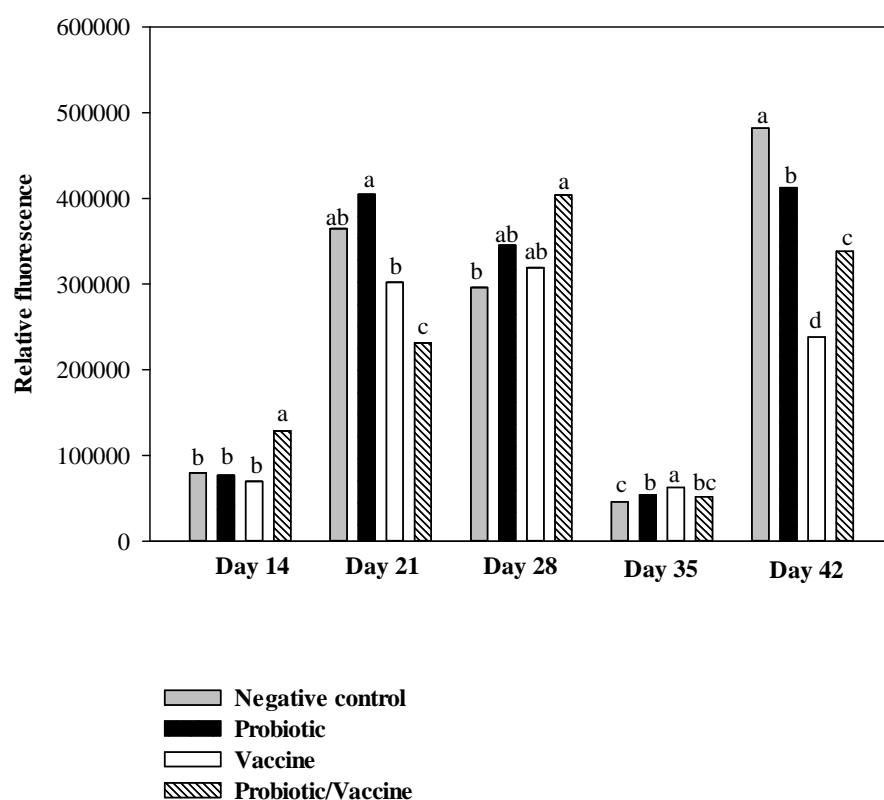


Figure 4.4. Oxidative burst of stimulated heterophils isolated from broilers fed an industry-based diet in combination with probiotic administration, coccidiosis vaccine administration, or a probiotic + vaccine on 14, 21, 28, 35 and 42 d of age. Each bar represents the mean (n=4). Means within each d with different letters differ significantly ($p \leq 0.05$).

Figure 4.5 shows the oxidative burst activity from PMA-stimulated monocytes. Monocyte oxidative burst was elevated ($p \leq 0.05$) in the probiotic + vaccine combination broilers on all d except 42 as compared to the control group. Furthermore, on d 21, an increase ($p \leq 0.05$) in monocyte oxidative burst was observed in vaccine alone broilers relative to the control group. Greater ($p \leq 0.05$) monocyte oxidative burst levels were seen in all groups on d 35 relative to the control group.

Figure 4.5.

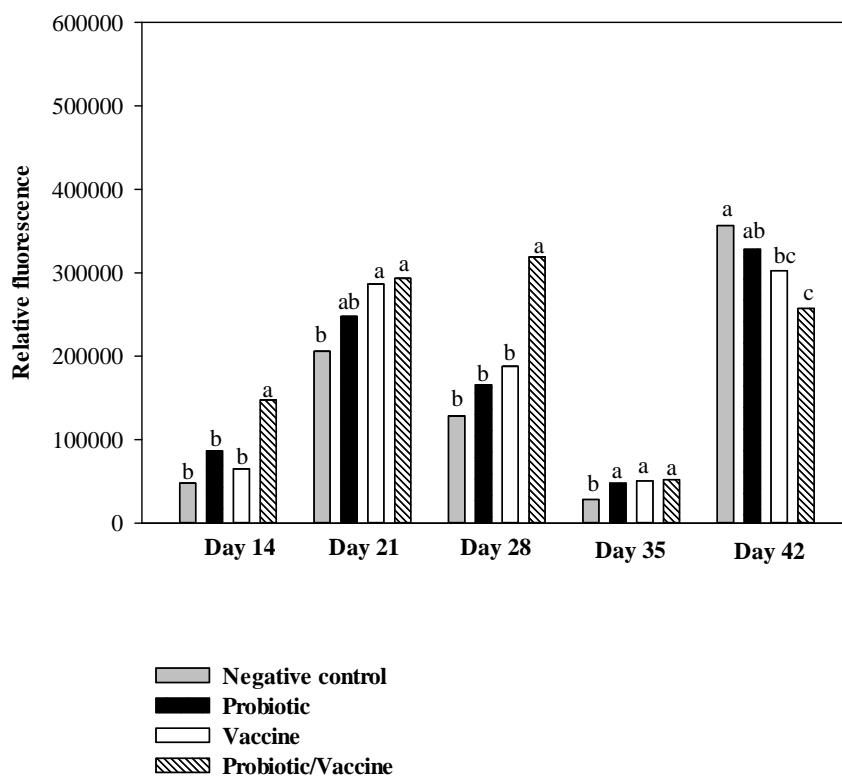


Figure 4.5. Oxidative burst stimulated monocytes isolated from broilers fed an industry-based diet in combination with probiotic administration, coccidiosis vaccine administration, or a probiotic + vaccine on 14, 21, 28, 35 and 42 d of age. Each bar represents the mean (n=4). Means within each d with different letters differ significantly ($p \leq 0.05$).

Clonal expansion of Con A-stimulated lymphocytes was measured using a colorimetric plate reader as shown in Figure 4.6. Lymphocyte proliferation was greater ($p \leq 0.05$) on d 14 and 42 in probiotic alone broilers and in all groups on d 21, when compared to controls. Increases ($p \leq 0.05$) in lymphocyte proliferation were also seen in probiotic + vaccine combination broilers on d 14.

Figure 4.6.

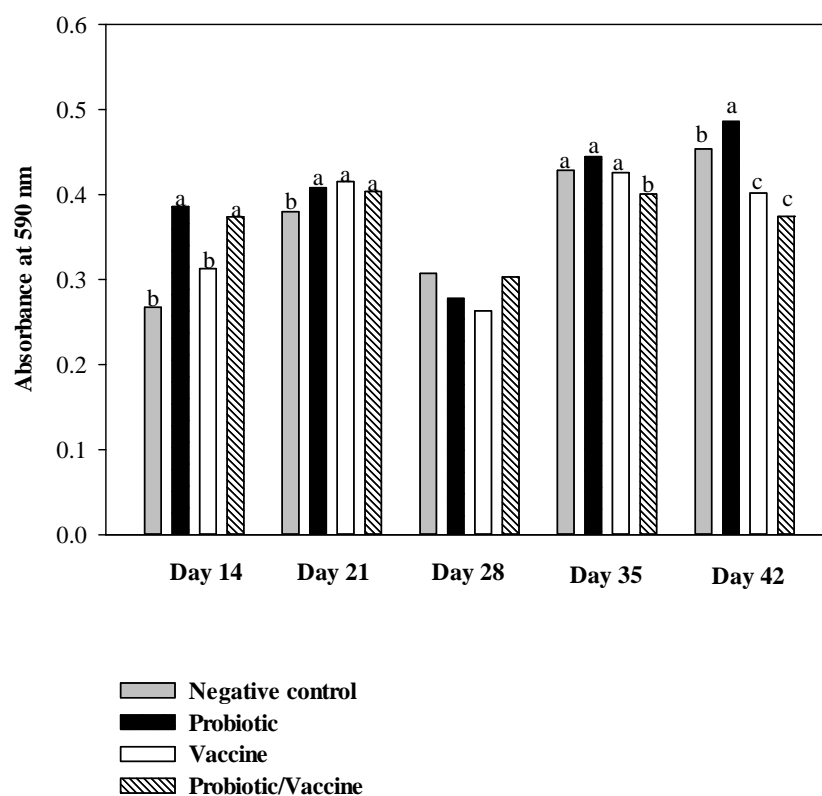


Figure 4.6. Proliferation of lymphocytes isolated from broilers fed an industry-based diet in combination with probiotic administration, coccidiosis vaccine administration, or a probiotic + vaccine on 14, 21, 28, 35 and 42 d of age. Each bar represents the mean ($n=4$). Means within each d with different letters differ significantly ($p \leq 0.05$).

Discussion

Oxidative burst of heterophils and monocytes was used as an assessment of innate immune function in this study. The term oxidative burst refers to the production of free radicals, including superoxide anions and nitric oxide, produced by stimulated phagocytes (Rose et al., 1995; Abbas and Lichtman, 2003). These products have been shown to be toxic and are generated during parasitic invasion by the host's innate cellular immune systems (Adams et al., 1990; Oswald et al., 1994). Allen (1997) conducted an experiment that measured the production of nitric oxide during an *Eimeria tenella* infection in chickens. A significant increase was found in plasma $\text{NO}_2^- + \text{NO}_3^-$ levels at 6 d post inoculation during primary infections with *Eimeria acervulina*, *tenella*, and *maxima*. Their findings may suggest an increase in monocyte and heterophil activity within the first week of *Eimeria* infection or coccidiosis vaccination.

Lymphocyte proliferation was used as a measure of cell-mediated immunity. Janeway and colleagues (2008) defines cell-mediated immunity as any adaptive immune response in which antigen-specific T-cells are the primary effector cell. Cell-mediated immunity is considered the primary protective mechanism in avian coccidiosis (Lillehoj, 1998; Lillehoj and Dalloul, 2004). Probiotic administration has been suggested to reduce intestinal inflammation by decreasing T-cell production and their production of cytokines (Lillehoj and Dalloul, 2004).

In Trial 1, a significant increase in heterophil oxidative burst was only observed on d 15 when compared to other treatments. However, in Trial 2, both heterophil and oxidative burst were increased on d 14. Taken together, the result of this treatment is

consistent with our previous investigation where heterophil oxidative burst was significantly greater on d 14 (Stringfellow et al., 2011). Results of heterophil upregulation have also been reported in broilers fed a *Bacillus*-, *Lactobacillus*- or *Bifidobacterium*-based probiotic (Farnell et al., 2006). Each individual treatment significantly increased heterophil and monocyte oxidative burst and lymphocyte proliferation at multiple time points in this study. Vaccination alone resulted in a significantly higher heterophil and monocyte oxidative burst at two time points (d 40 and 49) when compared to other treatments.

Limited studies have investigated the effect of probiotic administration to vaccinated broilers following an *Eimeria* challenge, therefore experiment two was designed to evaluate this effect. As previously mentioned, the combination treatment elicited an increase in both heterophil and monocyte oxidative burst in both trials on d 14 prior to any *Eimeria* oocysts challenge. Increases in the combination treatment were also observed on d 28 in monocyte oxidative but not at any other time point, when compared to the negative control. While we only observed significant effects of probiotic and vaccination within first 14-28 d, this may effectively enhance early production and overall health of chickens infected with coccidiosis. It's also important to note that both vaccine treatments resulted in a decreased immune response when compared to the negative control and probiotic (except monocyte oxidative burst) treatment, on d 42 of each of the immune assays.

Collectively, these data indicate that probiotic administration to broilers vaccinated against coccidiosis may prime the innate immune system to improve

pathogen protection. The administration of probiotics may be beneficial in enhancing mucosal immunity and improving the host's resistance to *Eimeria* spp. Due to the recent withdrawals of in-feed anticoccidial drugs and the high cost associated with *Eimeria* infections, the need for improved vaccine programs for broilers is increasingly important.

CHAPTER V

IMMUNOMODULATORY EFFECTS OF PROBIOTICS ON ANTICOCIDIAL MEASURES IN BROILERS

Introduction

Coccidiostats have been used in the poultry industry since the 1950's with the introduction of sulpha-drugs (McDougald, 1998). This discovery was subsequently followed by the discovery of several chemical and ionophore based coccidiostats. Aside from its vital role in the success of the poultry industry, some degree of resistance exists for all anticoccidial drugs currently used in the U. S. (McDougald, 1990; Williams, 2006). One alternative to anticoccidial drugs is the use of live *Eimeria* vaccines. Live oocyst coccidiosis vaccines consist of the oral delivery of small numbers of live *Eimeria* oocysts, which induces immunity to homologous challenge following vaccination (Allen and Jenkins, 2005). However, this method of control has not been universally accepted by the U. S. poultry industry for meat-producing birds due to the potentially negative effects on bird performance when compared with prophylactically medicated birds (Williams, 2002).

Avian immune responses to parasitic infections comprise a myriad of intestinal cellular and humoral immune mechanisms which rely on a balanced microbial population and a healthy intestinal tract (Lillehoj and Trout, 1996). Many factors associated with broiler production including transportation to the growing site,

overcrowding, vaccination or extreme temperatures may stress the chicks causing a disruption of the intestinal microflora and body defense mechanisms (Jin et al., 1997). Several broiler studies have reported that probiotic administration improves body weight gain, feed conversion and modulates gut mucosal immunity (Jin et al., 1997; Yeo and Kim, 1997; Dalloul et al., 2005, 2006). Modes of action of probiotics in poultry include maintaining a beneficial microbial population in the alimentary tract (Fuller, 1989), improving feed intake and digestion (Nahashon et al., 1992), and altering bacterial metabolism (Cole et al., 1987).

Previously we have shown that probiotic administration modulates the immune response of challenged (field strain *Eimeria* oocysts) and unchallenged broilers vaccinated with a live coccidiosis vaccine (Stringfellow et al., 2011). The present work was conducted to further evaluate these observations including a comparison with broilers receiving a medicated diet. Thus, we evaluated oxidative burst of heterophils and monocytes along with lymphocyte proliferation to investigate affects on leukocyte function during a 43 d pen study. We hypothesized that the oral administration of probiotics will modulate the immune response of broilers vaccinated with live *Eimeria* oocysts or broilers receiving an ionophore during an *Eimeria* infection.

Materials and Methods

Birds and Treatments

A total of 2,400 Cobb 500 male broiler chicks were obtained from a local commercial hatchery. Treatments consisted of a negative control, probiotic, medicated, vaccine, probiotic + vaccine combination and probiotic + medicated combination group.

Fifty chicks were placed in each pen (8 pens per treatment), achieving a final rearing density of 0.8 ft² (740 cm²)/bird with a 50:50 mixture of fresh and used litter in each pen. Birds were provided water, age appropriate supplemental heat (as needed), and fed a corn-soy diet formulated from current industry nutrient specifications. The feeding program was as follows: starter phase (D 1-14), grower phase (D 15-28), finisher phase (D 29-35) and withdrawal phase (D 36-42). These studies were conducted according to approved guidelines of the Texas A&M University Institutional Animal Care and Use Committee.

Experimental Design

An ANOVA designed experiment was conducted to evaluate the effect of probiotic administration and its ability to modulate immune function in broilers to coccidiosis vaccination. Peripheral blood was collected on d 6, 22, 36 and 43. Following blood collection, heterophils and mononuclear leukocytes from 10 broilers were collected and combined into three pooled (3-4 birds per sample) samples from each pen per treatment. To evaluate innate immunity, an oxidative burst assay was conducted on heterophils and monocytes using a fluorescent plate reader and an indicator of reactive oxygen species. Adaptive immunity was measured with a lymphocyte proliferation assay, which measured the ability of lymphocytes placed in short term tissue culture to undergo a clonal proliferation when stimulated *in vitro* by a mitogen.

Eimeria Infection

Field strain *Eimeria* isolates from our laboratory (College Station, TX) were used to challenge the birds in this study. *Eimeria* species in the challenge inoculum included

Eimeria acervulina, *E. tenella*, *E. maxima* and *E. mivati*. To induce a clinical infection, all birds received a feed challenge of 55,000 oocysts per bird on d 14 and a subsequent feed challenge on d 36 of 750,000 oocysts per bird.

Probiotic Administration

The probiotic, Biomin® PoultryStar (Biomin GmbH, Herzogenburg, Austria), contained 2.3×10^{12} cfu/kg of lactic acid producing bacteria including: *Enterococcus faecium*, *Pediococcus acidilactici*, *Bifidobacterium animalis* and *Lactobacillus reuteri*. The probiotic was administered through the drinking water at a concentration of 20 g/1000 birds per d which delivered 1×10^8 cfu/bird per d. Probiotic administration occurred on d 0-3, 8-10, 13-15, 27-29 and 34-36 per manufacturer's recommendations. The assumption was made that all birds drank similar amounts of water. The authors adjusted the dosage of the probiotic according to the commercial weekly water consumption rates of broilers (North and Bell, 1990).

Vaccination and Ionophore

Birds in the vaccinated groups received Coccivac®-B (Intervet/Schering-Plough Animal Health, Summit, NJ) using a commercial spray cabinet (Spraycox® II; Intervet/Schering-Plough Animal Health) on d of hatch. Chicks were allowed to preen under elevated light intensity for at least one hour prior to placement. Medicated diets contained the ionophore monensin (Coban®-60; Elanco Animal Health, Greenfield, Indiana) at manufacturer's recommended inclusion rates in all rations except the

withdrawal ration. Birds received probiotic continuously throughout the entire trial to comply with the manufacturer's recommendations and removed on d 36.

Leukocyte Isolation

Peripheral blood was collected by decapitation on 6 d of age only or venipuncture, and EDTA (Sigma-Aldrich, St. Louis, MO) was used as an anticoagulant. Blood was pooled from 10 birds per treatment (n = 3 separate pools/gradients per treatment). The polymorphonuclear and mononuclear cell fractions were isolated as previously described (Kogut et al., 1995). Briefly, blood was mixed with 1 % methylcellulose (Sigma-Aldrich), dissolved in Roswell Park Memorial Institute (RPMI)-1640 media (Mediatech Inc., Herndon, VA) at a ratio of 1:1.5 and centrifuged at 37 x g for 15 min. The supernatant was removed and re-suspended in Hank's balanced salt solution without calcium or magnesium (Mediatech Inc.) at a ratio of 1:1. The suspension was layered over a 1.077/1.119 Histopaque (Sigma-Aldrich) discontinuous gradient and centrifuged at 235 x g for 60 minutes. The suspension above the 1.077 Histopaque was collected for the mononuclear cell fraction and the interface of the two gradients was collected for polymorphonuclear cells. Cell types were then separated into conical tubes and washed with 40 mL of RPMI-1640 by centrifugation at 603 x g for 15 min. Following the RPMI-1640 wash, a complete solution of RPMI-1640 was added to reconstitute the cells. The media consisted of 5% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA); 4 mM L-glutamine, 10,000 U penicillin/mL, 10 mg streptomycin/mL (JR Scientific Inc., Woodland, CA); 1mM sodium pyruvate (Mediatech Inc.) and 0.1 mM non essential amino acid (MP Biomedicals, LLC, Solon,

OH). All agonists and substrates were suspended in this complete RPMI-1640. The cells were then quantified using a Neubauer hemacytometer (Electron Microscopy Sciences, Hatfield, PA); the stock was adjusted to a working concentration of 1×10^7 cells/mL and stored on ice until used. Cell viability was determined by a commercially prepared trypan blue solution (Sigma-Aldrich). Heterophil preparation obtained by this method was typically >95% pure and >95% viable (Kogut et al., 1995). Although the authors significantly enriched for lymphocytes, a pure culture of these cells were not obtained. The proliferation assay described below measured the expansion of viable cell numbers which resulted in an increase in the activity of the mitochondrial dehydrogenases in the sample. Monocytes are non-proliferative cells; therefore, only the lymphocytes in the culture should have proliferated and produced a significant signal.

Oxidative Burst Assay

Oxidative burst activity of heterophils and monocytes was measured using a Wallac fluorescent plate reader (Perkin Elmer, Boston, MA) and an indicator of reactive oxygen species, 2,7-dichlorofluorescein (**DCF-DA**; Sigma-Aldrich) as previously described (Xie et al., 2002). A minimal amount (90 μ L) of ethanol was used to dissolve the DCF-DA. Cells (1 mL) were preincubated for 30 minutes with 125 μ L of phorbol-12-myristate-13-acetate (**PMA**; 20 μ g/mL, Calbiochem, La Jolla, CA) at 42°C in a water-jacketed 5% CO₂ incubator. An equivalent volume of complete RPMI-1640 was added for the negative control treatments. Immediately after the preincubation period, 125 μ L of DCF-DA (0.2 mg/mL) was added and samples were mixed and aliquoted (8

replicates per sample) into a clear 96-well flat-bottomed plate. Oxidative burst was then measured at an excitation/emission wavelength of 485/530 nm.

Lymphocyte Proliferation Assay

A modified cell proliferation assay (Pauly et al., 1973) was conducted to evaluate cell mediated immunity. Concanavalin A (**Con A**; 20 µg/mL, Sigma-Aldrich) was used as a mitogen and (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (**MTT**; Sigma-Aldrich; 5mg/ mL) was used as a colorimetric substrate to analyze cell proliferation. Briefly, 75 µl of the mononuclear cell fraction was mixed with an equal volume of Con A, suspended in complete RPMI, inside a clear 96-well, flat bottomed plate for 24 hours at 42°C in a water-jacketed 5% CO₂ incubator. An equivalent volume of complete RPMI-1640 was added in replacement of the Con A for the negative control treatments. Following incubation, 15 µL of MTT was added to the cells and incubated for 4 hours at 42°C. Immediately after incubation 150 µL of MTT solvent (Sigma-Aldrich; 0.1 N HCL in anhydrous isopropanol) was added to the cells to dissolve the MTT formazan, resulting in a color change. The color change was measured at 450 nm by a colorimetric plate reader (Sunrise, Tecan, Austria).

Statistical Analysis

Statistical analysis was completed with the SPSS statistical software package (Chicago, IL). Data in all experiments were analyzed via a one-way ANOVA using the GLM procedure due to interactions between probiotic administration and coccidiosis vaccination, which were observed within all assays at multiple time points. Differences were deemed significant at $P \leq 0.05$ and means were separated using Duncan's multiple

range test. Pooled blood from the chickens of each treatment served as the experimental unit for these studies.

Results and Discussion

Figure 5.1 shows the oxidative burst activity from PMA-stimulated heterophils. On d 6, heterophil oxidative burst was increased ($p \leq 0.05$) in the vaccine alone group, vaccine with probiotic group, and the ionophore with probiotic group, when compared to the negative control. Consistent with results from our previous studies where samples were collected from unchallenged birds on d 14 and 15; the oxidative burst from the vaccine + vaccine broilers was significantly higher when compared to all other treatments. It's likely that probiotic administration created a more balanced and healthy intestinal microflora. Dalloul and colleagues (2005) concluded that probiotic administration enhance the mucosal immune response. Another additive effect of probiotic administration can be observed on d 22 and 43, (8 and 7 d post-challenge, respectively) in the probiotic + medication group. Heterophil oxidative burst was only increased ($p \leq 0.05$) in this group when compared to all other treatments, possibly as a result of established immunity in the other treatments from previous *Eimeria* oocyst exposure in the litter in addition to the removal of the ionophore on d 36. The exact mechanism behind this effect is unclear; it could be attributed to modulation of the gut environment and stimulation of the immune system by the probiotic (Koenen et al., 2004; Farnell et al., 2006; Stringfellow et al., 2011). It must be noted that during an elevated immune response, nutrients may be diverted from growth to immune cell development and function (Mountzours et al., 2010). The increased heterophil oxidative

burst observed on d 6 and 22 may facilitate early protection against parasitic invasion but infected birds will be allowed an opportunity for compensatory growth (Zhan et al., 2007). As such, an elevated immune response in the late days of broiler production may not award sufficient time for this process to occur.

Figure 5.1.

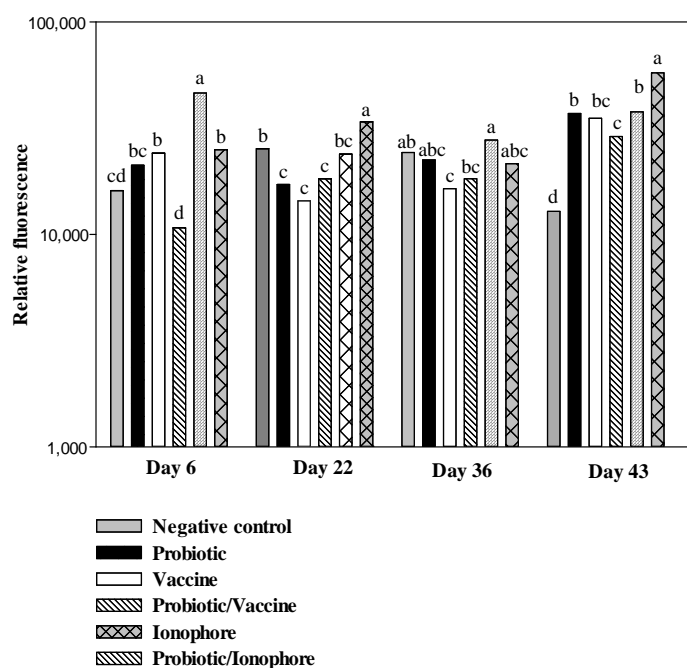


Figure 5.1. Oxidative burst of stimulated heterophils isolated from broilers fed an industry-based diet in combination with probiotic administration, coccidiosis vaccine administration, or a probiotic + vaccine on 6, 22, 36 and 43 d of age. Each bar represents the mean (n=4). Means within each d with different letters differ significantly ($p \leq 0.05$).

When oxidative burst activity was analyzed from PMA-stimulated monocytes (Figure 5.2) and Con A-stimulated lymphocytes (Figure 5.3), an additive affect elicited by probiotic administration to vaccinated broilers was only observed on d 36 in figure

5.1. When probiotic was combined with the ionophore, lymphocyte proliferation was greater ($p \leq 0.05$) on d 22 and 36 in the ionophore with probiotic group, when compared with all other treatments. Increases ($p \leq 0.05$) in lymphocyte proliferation were also seen in the vaccine alone and the ionophore alone group on d 43 in comparison with the negative control.

Figure 5.2.

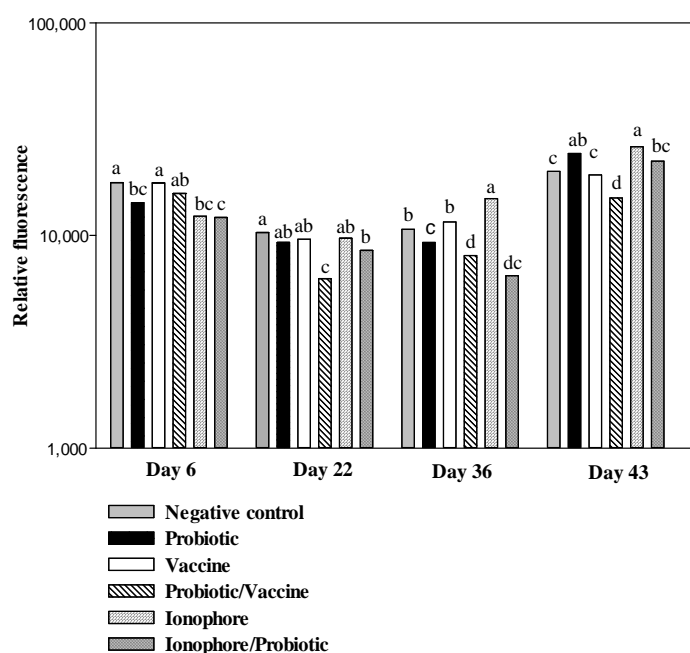


Figure 5.2. Oxidative burst of stimulated monocytes isolated from broilers fed an industry-based diet in combination with probiotic administration, coccidiosis vaccine administration, or a probiotic + vaccine on 6, 22, 36 and 43 d of age. Each bar represents the mean ($n=4$). Means within each d with different letters differ significantly ($p \leq 0.05$).

Figure 5.3.

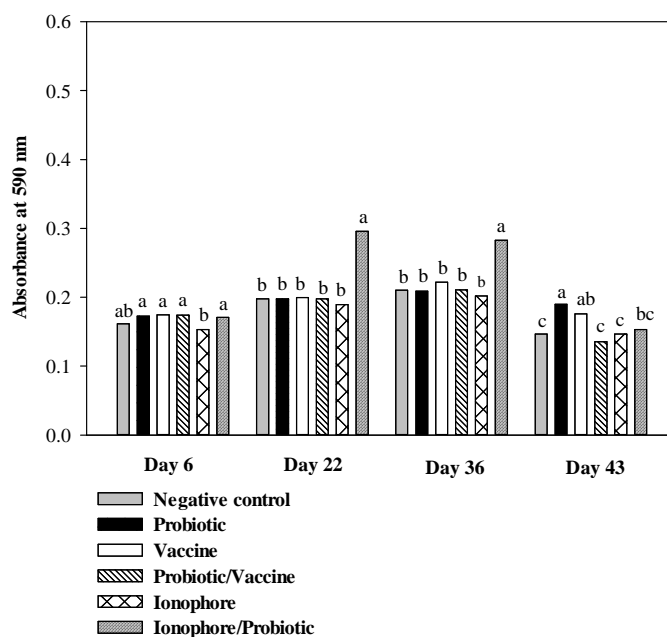


Figure 5.3. Proliferation of lymphocytes isolated from broilers fed an industry-based diet in combination with probiotic administration, coccidiosis vaccine administration, or a probiotic + vaccine on 6, 22, 36 and 43 d of age. Each bar represents the mean (n=4). Means within each d with different letters differ significantly ($p \leq 0.05$).

A growing body of evidence suggests beneficial health and immunomodulatory activity by consumption of probiotic bacteria in poultry. Probiotic bacteria are defined as live microorganisms which when administered to a host at the appropriate dose and time point has a beneficial influence on intestinal balance (Salminen et al., 1998), gut mucosal barrier (Salinen et al., 1996), and mucosal immune response (Koenen et al., 2004; Dalloul et al., 2004, 2006; Farnell et al., 2006; Stringfellow et al., 2011). Due to an increasing consumer preference for the removal of drugs in animal feed and the

perceived negative effects associated with coccidiosis vaccines, the present investigation compared the effects of probiotic administration in medicated and vaccinated broilers.

In summary, our results showed that probiotic administration to vaccinated or medicated broilers are capable of elevating heterophil and oxidative burst and lymphocyte proliferation at various time points. These findings suggest that simultaneous probiotic and vaccine or medication administration can improve responsiveness during a period of clinical coccidiosis.

CHAPTER VI

EFFECT OF PROBIOTIC ADMINISTRATION ON AVIAN-BETA DEFENSIN EXPRESSION IN VACCINATED BROILERS

Introduction

Avian coccidiosis, one of the most prevalent enteric diseases in commercial poultry production, is caused by host-specific protozoal parasites of the *Eimeria* genus (Chapman, 2001). Nine species of *Eimeria* have been described in chickens, but only seven of those species have been found to be pathogenic: *Eimeria acervulina*, *E. brunetti*, *E. maxima*, *E. tenella*, *E. praecox*, *E. mivati* and *E. necatrix* (Mcdougald, 2003). Current control measures include the use in-feed anticoccidial drugs or live vaccines (Vermeulen et al., 2001). Increasing reports have indicated that *Eimeria* strains have become resistant to anticoccidial drugs and therefore vaccination may be the only practical alternative to control coccidial infections in the field (Chapman et al., 2002.). Vaccines can effectively protect broilers by stimulating the immune system to respond to subsequent exposures to homogenous *Eimeria* species. Due to concerns of the timely onset of protective immunity and the risk of an *Eimeria* infection, coccidiosis vaccines have not been universally accepted for use in broiler production (Vermeulen et al., 2001).

Several mechanisms of action have been proposed by which probiotics may enhance mucosal protection against enteric infections and increase protection within the early days following vaccination and throughout the production cycle. Probiotic bacteria create beneficial microflora through competitive exclusion, improving digestion and by

changing bacterial metabolism (Jin et al., 1997). Furthermore, probiotic administration induces a myriad of physiological effects on the avian immune system including reducing *Eimeria* oocysts shedding (correlates to increased resistance), increasing gamma interferon and IL-2 secretion (Dalloul et al., 2005) and increasing phagocytic oxidative burst levels (Farnell et al., 2006; Stringfellow et al., 2011).

Probiotic administration was demonstrated to upregulate human β -defensin genes in mammalian cells (Wehkamp et al., 2004). Defensins are a family of cysteine-rich antimicrobial peptides that belong to the innate immune system (Sugiarto and Yu, 2006). Two major classes of defensins exist; α -defensins (found only in mammals) and β -defensins (found throughout all vertebrate species) (Lehrer et al., 2002; Lynn et al., 2007). Avian β -defensins have a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, eukaryotic parasites and enveloped viruses (Hancock, 2001). The antimicrobial mechanism of AVBD has not been proposed; however β -defensins in humans and mammals have been speculated to cause membrane disruption of invading microbes via a carpet wormhole model which ultimately results in self-promoted uptake of the protein (Kagan et al., 1990; Hancock, 1997; Ganz, 2003; Satchell et al., 2003). In mammals, defensins are highly represented and constitutively expressed in blood and epithelial cells lining the mucosal surfaces of the respiratory, digestive and urogenital tracts (Bals et al., 1999). Currently, 13 different AVBD have been reported to be expressed in a variety of tissues including all mucosal surfaces (Xiao et al., 2004; Lynn et al., 2007). Due to their close proximity to mucosal surfaces, modulation of these proteins with probiotic bacteria may promote a healthy

gastrointestinal environment and improve defense mechanisms. Research investigating the modulation of AVBD genes is limited, several AVBD genes have been shown to increase in response to lipopolysaccharides and *Salmonella* Enteritidis (Yoshimura et al., 2006; Mageed et al., 2007), in hen vaginal tissue and *Haemophilus paragallinarum* (Zhao et al., 2001) in tracheal tissue.

To date, the authors found no research analyzing the effect of probiotic administration on AVBD expression in broilers vaccinated with a live commercial coccidiosis vaccine. Thus, the objective of this study was to orally vaccinate d old broilers, and administer probiotics in the drinking water and collect tissue from the liver and ileum to evaluate AVBD 2 and 9 gene expression. We hypothesized that probiotic administration will modulate defensin expression in broilers vaccinated against coccidiosis.

Materials and Methods

Birds and Treatments

A total of 400 Cobb male broiler chicks were obtained from a local commercial hatchery. Treatments consisted of a negative control, probiotic, vaccine, and probiotic + vaccine combination. Fifty chicks were placed in each pen (1 pen per treatment), achieving a final rearing density of 0.8 ft² (740 cm²)/bird with fresh pine shavings in each pen. Birds were provided water, age appropriate supplemental heat (as needed), and fed a corn-soy diet formulated from current industry nutrient specifications. Birds were fed a starter diet throughout the termination of the study. This study was

conducted according to approved guidelines of the Texas A&M University Institutional Animal Care and Use Committee.

Experimental Design

The probiotic, Biomin® PoultryStar (Biomin GmbH, Herzogenburg, Austria), contained 2.3×10^{12} cfu/kg of lactic acid producing bacteria including: *Enterococcus faecium*, *Pediococcus acidilactici*, *Bifidobacterium animalis* and *Lactobacillus reuteri*. The probiotic was administered through the drinking water at a concentration of 20 g/1000 birds per d which delivered 1×10^8 cfu/bird per d. Probiotic administration occurred on d 0-2, 8-10 and 15-17 per manufacturer's recommendations. The assumption was made that all birds drank similar amounts of water. The authors adjusted the dosage of the probiotic according to the weekly water consumption rates of broilers (North and Bell, 1990). Birds in the vaccinated groups were individually vaccinated with Coccivac®-B (Intervet/Schering-Plough Animal Health, Summit, NJ) via an oral gavage needle on d of hatch.

Defensin Expression

Birds (n=10) were sacrificed on d 21 by carbon dioxide and ileum and liver tissue were rinsed with cold, sterile saline and suspended in RNA-later and stored at -20°C for subsequent RNA isolation. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Samples were then resuspended in RNA-ase free water and the total RNA concentration and purity was determined spectrophotometrically (Nanodrop ND-1000; NanoDrop Technologies, Wilmington, DE). Samples were then diluted to 2µg RNA/µl and DNA-ase treated

(Invitrogen) before first strand cDNA synthesis. The RNA samples were then reverse transcribed using SuperScript III First-Strand Synthesis (Invitrogen) per manufacturer's recommendations and the amplification products were verified by gel electrophoresis (1%). Following reverse transcription, gene expression was analyzed using a TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA). Probes were designed with GenBank Accession No. AF033336 (AVBD 2) and No. AY621311 (AVBD 9) and GAPDH (NM204305) was chosen as the internal reference. The reaction mixture contained 1 μ l of 20X TaqMan gene expression assay, 10.0 μ l of 2X TaqMan gene expression master mix (Applied Biosystems), 50 ng of cDNA template and the volume was adjusted to 20 μ l with nuclease-free dH₂O. Reactions were run in triplicate in 384-well plates in a final volume of 20 μ l. Each PCR run consisted of a 10-min hot start at 95°C, which activated the conjugated polymerase, followed by 40 cycles consisting of 15 s of denaturation at 95°C, 1-min of annealing at 60°C, and a fluorescent read step using the Applied Biosystems 7900HT Real-Time PCR System.

Statistical Analysis

Using the comparative CT method described in the ABI Prism 7700 Sequence Detection System User Bulletin no. 2.; Applied Biosystems. Statistical significance among four different groups was analyzed using a student's T-test after CT values were normalized to GAPDH. Statistical analysis was completed with the SPSS statistical software package (Chicago, IL).

Results and Discussion

The presence of ionophore-resistant field strains of *Eimeria* has stimulated increased research toward developing alternative methods of preventing coccidiosis in broilers (Allen and Fetterer, 2002). Live coccidiosis vaccines are currently being used by broiler producers, however growers are concerned with the risk of a low level infection, slow onset of immunity and negative effects on performance (Williams et al., 2002). Live vaccination is based on the well-established concept that chickens infected with *Eimeria* oocysts rapidly develop protective immunity against a homologous challenge (Shirley et al., 2005). Host immunity is of primary concern in determining both primary and subsequent infections with *Eimeria* parasites (Lillehoj and Trout, 1996; Shirley et al., 2005; Yun et al., 2000). As a result, there has been increased interest in developing novel strategies to extrapolate the immunobiology of host protective mechanisms in the intestine (Yun et al., 2000). Several studies have demonstrated that probiotics elicit disease prevention and immune enhancement however, the authors found no research reporting their effect on live coccidiosis vaccines.

Defensins are cysteine-rich antimicrobial peptides that have been demonstrated to possess antimicrobial activity against protozoa (Donovan and Topley, 2003). In

addition to its antimicrobial activity, defensins have been suggested to modulate humoral immunity and antibody titers following infectious bursal disease virus vaccination (Yurong et al., 2006). Microarray data from our laboratory reported that AVBD 2 and 9 genes are highly expressed in these tissues (Li et al., 2008). The present study evaluated the effect probiotic administration on AVBD 2 and 9 gene expression in the liver and ileum of broilers vaccinated with live *Eimeria* oocysts. The results showed that liver AVBD 2 expression significantly increased in the probiotic + vaccine group relative to the probiotic alone group (Fig. 6.1). Avian β -defensin 9 was also demonstrated to have an increased expression in the vaccine group when compared to the control birds (Fig. 6.2). Little is known about the repertoire and biological functions of AVBD, however it's been suggested that modulation of their expression could be an alternative strategy to improve health and promote growth (Dijk et al., 2007). Several mammalian defensins have been demonstrated to link innate to adaptive immunity by recruitment of effector cells via chemotaxis (Yang et al., 1999). The present findings indicate that increased liver AVBD 2 and AVBD 9 expression might be associated with a host immune response to probiotic and vaccine administration in broilers.

Figure 6.1.

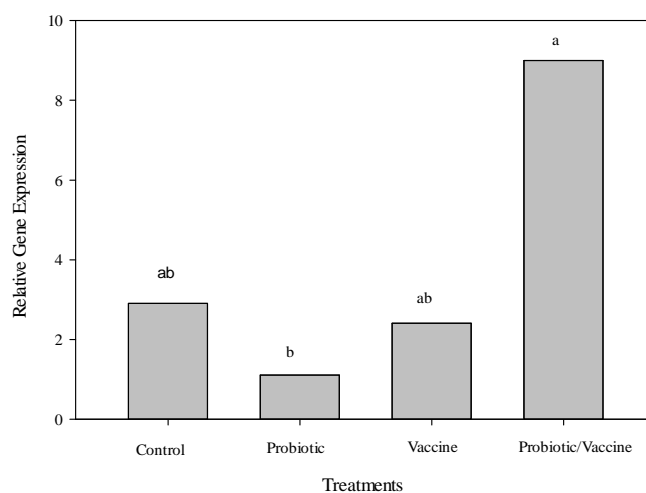


Figure 6.1. Relative expression of AVBD 2 from liver tissue of broilers fed an industry-based diet in combination with probiotic administration, coccidiosis vaccine administration, or a probiotic + vaccine on 21 d of age. Each bar represents the mean (n=6). Means within each d with different letters differ significantly ($p \leq 0.05$).

Figure 6.2.

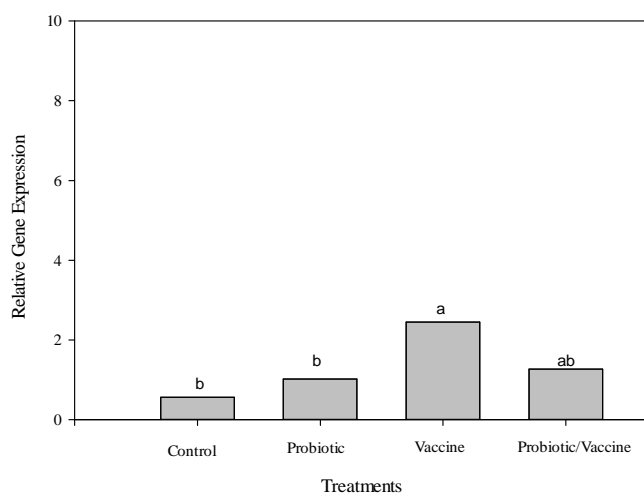


Figure 6.2. Relative gene expression of AVBD 9 from liver tissue of broilers fed an industry-based diet in combination with probiotic administration, coccidiosis vaccine administration, or a probiotic + vaccine on 21 d of age. Each bar represents the mean (n=6). Means within each d with different letters differ significantly ($p \leq 0.05$).

Several AVBD have been reported to have high to moderate gene expression in the small intestine of chickens (Lynn et al., 2004). Figure 6.3, shows the effect of probiotic administration on AVBD 2 in the ileum of 21 d old broilers however, differences in gene expression between treatments was not observed. Interestingly, all treatments resulted in decreased ($p \leq 0.05$) gene expression when compared to the control birds when AVBD 9 was evaluated (Fig. 6.4).

Figure 6.3.

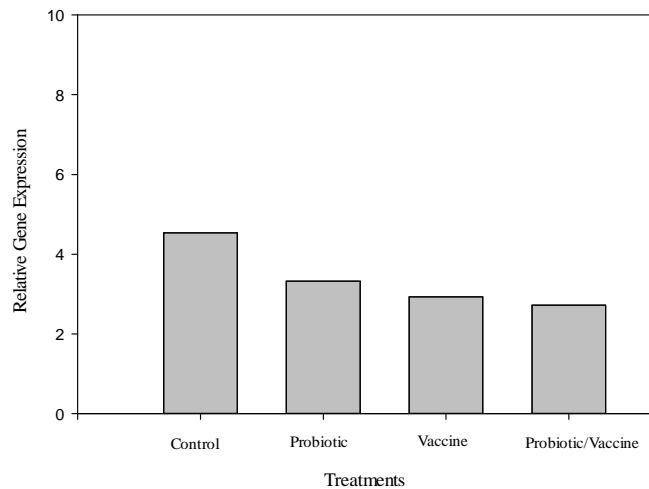


Figure 6.3. Relative gene expression of AVBD 2 from ileum tissue of broilers fed an industry-based diet in combination with probiotic administration, coccidiosis vaccine administration, or a probiotic + vaccine on 21d of age. Each bar represents the mean (n=6). Means within each d with different letters differ significantly ($p \leq 0.05$).

Figure 6.4.

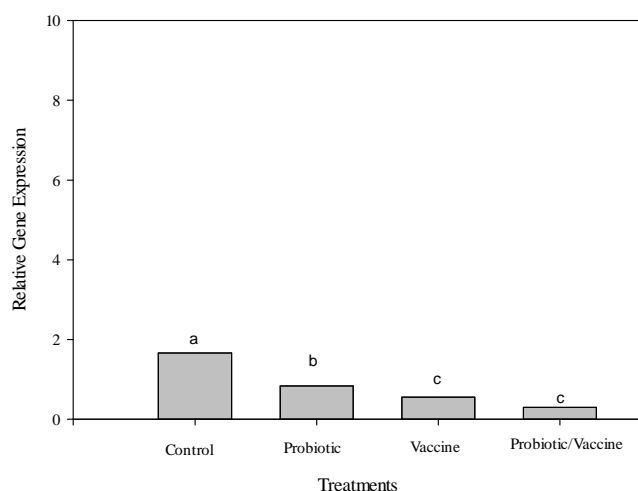


Figure 6.4. Relative gene expression of AVBD 9 from ileum tissue of broilers fed an industry-based diet in combination with probiotic administration, coccidiosis vaccine administration, or a probiotic + vaccine on 21d of age. Each bar represents the mean (n=6). Means within each d with different letters differ significantly ($p \leq 0.05$).

In addition to other non-specific defense barriers, defensins are essential components of the epithelial defense barrier that provides immediate protection against bacteria invasion conversely data reporting their significance on *Eimeria* has not been reported (Lillehoj et al., 2004). The differences in gene expression may act in facilitating pathogen clearance during an infection; AVBD 2 and 9 have both been suggested to act as antimicrobial agents (Yoshimura et al., 2006; Milona et al., 2007). The modulation of defensins found within the ileum in this study could play a chemotactic role for leukocytes following live coccidiosis vaccine administration via their ability to attract heterophils, monocytes, macrophages and T-cells to the site of infection (Sugiarto and Yu, 2006). Hancock and Diamond (2000), suggested that

defensins may also promote wound healing by recruiting fibroblast at inflammation sites. Fibroblasts are essential components in reepithelialization of damaged surfaces (Sugiarto and Yu, 2006). As such, modulating defensin gene expression with probiotic administration may counter any negative effects on performance as live *Eimeria* vaccines cause mild coccidiosis which is described by the presence of lesions and slowing of growth rate (Mathis and Lang, 2001).

Taken together, these data suggest that oral administration of probiotics can modulate AVBD 2 and 9 gene expression in broilers vaccinated with a live coccidiosis vaccine. To our knowledge, this is the first study demonstrating the gene expression of AVBD 2 and 9 in response to probiotic administration to broilers vaccinated against coccidiosis. Some are upregulated and some are downregulated, suggesting a diverse role within the bird.

CHAPTER VII

CONCLUSIONS

In chapter III, in experiment one, two studies were conducted to evaluate the immune response of broilers vaccinated with a live coccidiosis vaccine along with probiotic administration. The results from the first study showed that probiotic alone or in combination with the live coccidiosis vaccine caused significant increases in heterophil and monocyte oxidative burst and proliferation of lymphocytes at multiple time points. Interestingly, on d 14 of all the immune assays, the probiotic + vaccine combination treatment resulted in significant increases in comparison to all other treatments. The significance of this finding may indicate that probiotic administration may have improved gut function and the development of the intestinal immune system, priming the coccidiosis vaccine response, as the immune system of neonates is functionally immature within the first 14 d. Similar results were not seen in the second study, however probiotic alone and vaccine alone treatments were able to significantly increase immune responses when compared to with the negative control.

In chapter IV, in the first study, we only observed an additive effect of probiotic administration in vaccinated broilers only on d 15 of heterophil oxidative burst when compared to all other treatments. However, on d 15 of monocyte oxidative burst increases ($p \leq 0.05$) were seen among all treatment when compared with the negative control. Overall, the vaccine alone treatment was able to elevate ($p \leq 0.05$) the immune response on d 40 and 49 of heterophil and monocyte oxidative burst when compared to all other treatments. Similarly, on d 40 proliferation was significantly higher in the

vaccine alone treatment when compared to all other treatments. In the second study, probiotic administration to vaccinated broilers resulted in significantly higher heterophil and monocyte oxidative burst on d 14 when compared to all other treatments. Similar results were seen in Chapter III. Both probiotic treatments were also elevated ($p \leq 0.05$) lymphocyte proliferation on d 14. On d 42, all vaccine treatments had decreased ($p \leq 0.05$) immune responses when compared to the negative controls. This may be a positive affect as birds experiencing an immune response will not consume as much feed and as a result decreases weight and muscle mass. It may be more cost effective for birds to experience elevated immune responses early in the production cycle providing them with an opportunity for compensatory growth.

Chapter V was designed to compare the probiotic affects in vaccinated versus medicated broilers during a clinical *Eimeria* challenge. On d 6, an additive affect of probiotic administration to vaccinated broilers was observed in heterophil oxidative burst in comparison with all other treatments. Heterophil oxidative burst was also increased ($p \leq 0.05$) in the ionophore with probiotic group on d 22 and in all treatments on d 43. Monocyte oxidative burst was increased ($p \leq 0.05$) in the vaccine with probiotic group on d 36 and 43, compared to the negative control. An increase ($p \leq 0.05$) in monocyte oxidative burst was also observed on d 43 in the ionophore alone group. Notably, lymphocyte proliferation was greater ($p \leq 0.05$) on d 22 and 36 in the ionophore with probiotic group, when compared to all other treatments. Increases ($p \leq 0.05$) in lymphocyte proliferation were also seen in the vaccine alone and the ionophore alone group on d 43.

In chapter VI, the results showed that liver AVBD 2 gene expression increased ($p \leq 0.05$) in the probiotic + vaccine group relative to the probiotic alone group. Ileum AVBD 2 gene expression was not affected among any of the treatments evaluated. Liver AVBD 9 was demonstrated to have a higher ($p \leq 0.05$) gene expression in the vaccine group when compared to the control birds. Conversely, AVBD 9 gene expression in the ileum was significantly decreased all treatments with in comparison with the negative control groups. These data suggest that probiotics can modulate the immune response and may play a role in live coccidiosis vaccination in broilers.

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